Stimulation pulse characteristics and electrode configuration determine site of excitation in isolated mammalian skeletal muscle: implications for fatigue

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Cairns SP, Chin ER, Renaud J-M. Stimulation pulse characteristics and electrode configuration determine site of excitation in isolated mammalian skeletal muscle: implications for fatigue. J Appl Physiol 103: 359–368, 2007. First published April 5, 2007; doi:10.1152/japplphysiol.01267.2006.—We examined whether electrical field stimulation with varying characteristics could excite isolated mammalian skeletal muscle through different sites. Supramaximal (20-V, 0.1-ms) pulse stimulation with transverse wire or parallel plate electrodes evoked similar forces in nonfatigued slow-twitch soleus and fast-twitch extensor digitorum longus (EDL) muscles from mice. D-tubocurarine shifted the twitch force-stimulation strength relationship toward higher pulse strengths with both electrode configurations in soleus muscle, suggesting that weaker pulses excite muscle via neuromuscular transmission. With wire stimulation, movement of the recording electrode along the muscle caused a delay between the stimulus artifact and the peak of the action potential, consistent with action potential propagation along the sarcolemma. TTX abolished all contractions evoked with 20-V, 0.1-ms pulses, suggesting that excitation occurred via voltage-dependent Na+ channels and, hence, muscle action potentials. TTX did not prevent force development with ≥0.4-ms pulses in soleus or 1-ms pulses in EDL muscle. Furthermore, myoplasmic Ca2+ (i.e., the fura 2 ratio) and sarcomere shortening were greater during tetanic stimulation with 2.0-ms than with 0.5-ms pulses in flexor digitorum brevis fibers from rats. TTX prevented all shortening and Ca2+ release with 0.5-ms, but not 2.0-ms, pulses, indicating that longer pulses can directly trigger Ca2+ release. Hence, proper interpretation of mechanistic studies requires precise understanding of how muscles are excited; otherwise, incorrect conclusions can be made. Using this new understanding, we showed that disrupted propagation of action potentials along the surface membrane is a major cause of fatigue in soleus muscle that is focally and continuously stimulated at 125 Hz.

electrical field; skeletal muscle contraction; high-frequency fatigue; pulse parameters; muscle fiber type

FOR STUDY OF THE CONTRACTILE PERFORMANCE of skeletal muscle without complications arising from a variable motor drive from the central nervous system, contractions are often triggered peripherally using artificial electrical field stimulation. This may involve stimulation of nerve or muscle in situ (18, 20, 25, 30) or stimulation of several types of isolated muscle preparations (2, 6, 9, 10, 13, 26, 35). The methodology for such electrical stimulation varies considerably in terms of the stimulation characteristics, which include electrode configuration and pulse parameters. The electrode configurations may differ in terms of their physical form, e.g., wires, coils, or plates of various sizes (3–8), and their placement, e.g., parallel to the long axis (i.e., flanking) or transversing the muscle (3–8, 13, 21, 26, 34). In addition, the electrical field depends on the pulse strength and duration. It is often assumed that if stimulation is supramaximal for the twitch, then an action potential is triggered in the sarcolemma and all the normal physiological processes are involved, regardless of the stimulation characteristics. However, electrical stimulation could, in principle, excite muscle through different peripheral sites, e.g., nerve terminals or transverse (T)-tubular membranes. In consequence, this could have resulted in considerable misinterpretation of data in the literature concerned with the role of the action potential in muscle function. Clearly, a full understanding of the stimulation methodology is required, especially in studies undertaken to establish the site(s) of impairment during muscle fatigue, i.e., a reduced muscle force.

Few studies have carefully investigated the sites of muscle excitation with different electrode configurations. Nakajima and Gilai (32) elegantly demonstrated in amphibian muscle fibers that massive field stimulation with parallel plate electrodes triggers an action potential immediately in the surface membrane, which then propagates radially along the T-tubular membranes. In addition, they focally stimulated the sarcolemma with a single wire electrode and showed that an action potential propagates along the surface membrane. We previously made use of the ideas that transverse wire stimulation triggers a propagated action potential in the surface membrane, whereas plate stimulation triggers an action potential simultaneously along the length of the muscle (3, 8). In these experiments, the peak force depression and fade at lowered extracellular Na+ concentration ([Na+]o) were exacerbated with wire compared with plate stimulation (3), and fatigue with repeated short tetani occurred more rapidly with wire than with plate stimulation (8). Our interpretation of these data is that surface membrane propagation of action potentials is impaired when wire stimulation is used.

The effects of different stimulation pulse parameters on the sites of excitation are also not fully understood. It is well established that nerve and muscle action potentials require Na+ influx through voltage-dependent TTX-sensitive Na+ channels (3, 19, 37–39, 41). Action potentials are thought to be triggered...
more readily in nerve than in muscle membranes (18, 20, 30, 40), and recently the action potential threshold has been shown to be lower at the end plate, than at the end plate border, than in the extrajunctional membrane, respectively (31, 37, 40). Also, it appears from the twitch force-stimulation strength relationship that action potential threshold is higher in T-tubular (33, 35) than in surface (3, 6, 7, 11) membranes. We thus postulate that as the pulse strength or duration is increased, the site of action potential initiation would shift sequentially from the motor axon, to the end plate region, to along the surface membrane, and eventually to T-tubular membranes. Moreover, very long pulses may elicit multiple action potentials (11, 28–30) or directly depolarize the voltage sensor proteins of excitation-contraction coupling, i.e., dihydrotrypiridine (DHP) receptors, in the T-tubular membranes (6, 36, 39), leading to Ca$^{2+}$ release from the sarcoplasmic reticulum (SR).

Therefore, the main aim of this study was to determine the site(s) at which electrical field stimulation excites isolated mammalian skeletal muscle using 1) the most common electrode configurations, i.e., transverse wires or parallel plates, and 2) a range of stimulation pulse parameters. A second aim was to establish whether there were differences in excitation between fast- and slow-twitch muscles when some stimulation characteristics were used. Finally, with the new understanding from these findings, we sought to use different stimulation characteristics to examine potential sites of impairment during fatigue as a result of prolonged continuous tetanic stimulation.

**MATERIALS AND METHODS**

**Muscle Preparations**

For most experiments, whole muscle preparations from mice were used. In some experiments, single muscle fibers from rats were required to measure myoplasmic Ca$^{2+}$ concentration ([Ca$^{2+}$]) and sarcromere shortening. The study was approved by the Animal Ethics Committee of the University of Auckland, the Animal Care Committee of the University of Ottawa, and the Institutional Animal Care and Use Committee of Pfizer Global Research and Development.

Whole muscle preparation. Adult Swiss CD-1 mice of either sex (20–30 g body wt) were killed by cervical dislocation for the isometric force experiments (performed at the University of Auckland). For the action potential experiments (performed at the University of Ottawa), mice were anesthetized with pentobarbital sodium (0.8 mg/10 g body wt ip; Somontol, MTC Pharmaceuticals, Cambridge, ON, Canada) before muscle isolation and then killed by pentobarbital overdose. Intact slow-twitch soleus or fast-twitch extensor digitorum longus (EDL) muscles were dissected in the control solution and bubbled with 95% O$_2$–5% CO$_2$ at room temperature. Nerve twigs along its length were visible. Embedded in connective tissue entered the muscle about half-way into the muscle. In addition, two fine (0.65-mm-diameter) wire electrodes entered between the plates in a horizontal plane and then turned 90° to transverse the width of the muscle (5 mm long) in front of and behind the muscle. These wires were positioned about halfway along the length of the muscle and separated by 4–6 mm. Much of each wire electrode was covered with earthing sleeve and partly shielded from the plate electrodes with a Perspex attachment. Both electrode types could be removed if required. Rectangular wave pulses were initiated from a computer using a custom-written LabVIEW program and delivered to the stimulation electrodes via a purpose-built power amplifier (MOSFET). A switch on the amplifier permitted intracellular recordings of muscle action potentials without major interference by the stimulus artifact (3, 41).

In setup 2, a muscle was mounted vertically about midway between two rectangular (2 × 5 cm) plate electrodes, which flanked and engulfed the muscle (Fig. 1B). The plates were slid into a holder, which fixed them at 7.7 mm apart. In addition, two fine (0.65-mm-diameter) wire electrodes entered between the plates in a horizontal plane and then turned 90° to transverse the width of the muscle (5 mm long) in front of and behind the muscle. These wires were positioned about halfway along the length of the muscle and separated by 4–6 mm. Much of each wire electrode was covered with earthing sleeve and partly shielded from the plate electrodes with a Perspex attachment. Both electrode types could be removed if required. Rectangular wave pulses were initiated from a computer using a custom-written LabVIEW program and delivered to the stimulation electrodes via a purpose-built power amplifier (MOSFET). A switch on the amplifier permitted stimulation via wire or plate electrodes. The standard bipolar pulses (20 V, 0.1 ms) were supramaximal for the twitch. Further increasing the output from the stimulator resulted in the voltage across the electrodes saturating at a maximum of 26 V. Pulse strength was measured between dry electrodes because of electrical interference when solution was in the bath.

In setup 3, dissociated single FDB fibers were placed in a one-pass perfusion chamber designed for field stimulation (model RC-21BFRS, Warner Instruments). The chamber contained two parallel wire electrodes attached to the opposite sides of an oval-shaped bath (6.3-mm electrode separation), with glass coverslips used for the base and cover. Some fibers lay parallel to the electrodes, whereas others lay at angles. Rectangular pulses were produced with a Grass S88 stimulator and SIUS isolation unit, as described for setup 2. The stimulation
pulses (5 V, 0.5 ms) were supramaximal for twitch shortening. Pulse strength was measured in solution in the bath.

**Action Potential Measurements**

A whole muscle was mounted in a chamber (2 ml) that permitted flow of preheated oxygenated solution (15 ml/min) through it and then stretched to optimal length for the tetanus. Conventional glass microelectrodes filled with 3 M KCl were used to record intracellular action potentials in the surface membrane of single fibers in the surface layer of cells within soleus muscles using setup 1. The stimulation pulses used for most experiments are described elsewhere (3) and are the same as those used for the contractile studies. To test for action potential propagation along the surface membrane, we compared the time from the onset of the stimulus artifact to the peak of the action potential, i.e., the propagation time, when the action potential was recorded at various distances from the wire stimulating electrode (the cathode). The propagation time was determined as the average of two to five action potentials measured at each location. The recording microelectrode could readily be moved along the length of the muscle with a micromanipulator. The action potential profile could not be measured when the parallel electrode configuration in setups 2 and 3 was used because of considerable interference with large stimulus artifacts, i.e., an electrical signal arising from the stimulation electrodes that is not a muscle membrane response.

**Isometric Force Measurements**

The isometric force data were obtained only in setup 2. Briefly, one tendon of a whole muscle was attached to a force transducer (model KSP-2-E3, Kyowa) and the other was fixed as described previously (3, 6, 8). A muscle was bathed in ~100 ml of solution in a chamber, oxygenated from its base, and immersed in a temperature-controlled water bath. Temperature was maintained by an ultrathermostat (model 3, Haake, Karlsruhe, Germany) that was coupled to a refrigeration unit (model DK 12, Haake). Contractions were continuously monitored on a chart recorder, and selected contractions were recorded in digital form on a computer. The experimental protocol involved adjustment of muscle length until maximum tetanic force was achieved. Contractions were evoked every 5 min during equilibration for 30–60 min. Tetani were usually evoked at 125 Hz for 2 s in soleus and at 200 Hz for 0.5 s in EDL muscles. These stimulation frequencies (and durations) were required to generate maximum force but exceed the naturally occurring median firing frequencies of ~20 Hz in soleus and 50–90 Hz in EDL motor units of rodents (17). In some cases, even higher frequencies were used to test for mechanisms (see Fig. 5). Muscles with a rapid rundown of peak tetanic force, i.e., >0.2%/min, were rejected. Peak twitch or tetanic force was determined as the difference between the maximum force during a contraction and the baseline level. The force evoked with different electrode configurations was expressed by normalization of the peak force of contractions.
evoked with wires to that for bracketing contractions evoked with plates (setup 2). In preliminary experiments, the peak tetanic force evoked in soleus muscle with wires alone (20 V, 0.1-ms pulses) was slightly higher [103.1 ± 0.6% (SE), n = 6, P < 0.05] than that evoked by wires when both electrode configurations were present. The peak force evoked with plate electrodes alone was identical to that evoked when both electrode configurations were present (99.5 ± 0.3%, n = 8). The pulse strength at which maximum twitch force was first achieved was determined when the twitch force first exceeded 98% of the plateau force value. Stimulation strengths that generated 50% and 75% of maximum twitch force were determined by interpolation between points. The experiments at elevated extracellular K+ concentration ([K+]o) involved exposure to 10 or 11 mM K+ for ≥60 min until a steady-state tetanic force was achieved; then TTX was added to the bath. TTX was applied for up to 30–120 min at normal or elevated [K+]o, with tetanic stimulation every 5 min.

**Sarcomere Shortening and [Ca2+]i, Measurements**

Single dissociated fibers from rat FDB muscles lacked tendons and loosely adhered to the base of the chamber (setup 3). Fibers were superfused with oxygenated solution (1 ml/min). To assess contractile function, we measured changes of sarcomere length as an index of fiber shortening with a video-based system (Myocam, IonOptix). Fibers were illuminated with infrared light, and images were captured in digital form with a CCD100M camera using the IonOptix SarcLen data acquisition software. The light intensity of the I and A bands was analyzed by Fourier analysis, which gives one major peak; from the frequency of that peak, the sarcomere length was calculated using a calibration. Tetani were evoked at 30–100 Hz for 0.4 s. The sampling rate was 200 Hz.

FDB fibers were exposed to 5 mM fura 2-AM (Molecular Probes) dissolved in culture medium for 30 min at 37°C, transferred to the chamber used in setup 3 (263-μl volume), and superfused with physiological saline solution. Fura 2-loaded fibers were excited using a Hyperswitch (IonOptix) for dual excitation at 340 and 380 nm, with the fluorescence emission ratio at 510 nm being proportional to [Ca2+]i. The fura 2 ratio (340/380) represents [Ca2+]i. The sampling rate was 250 Hz. The peak fura 2 ratio during tetanic stimulation was calculated as the mean of all data points during the plateau phase of the Ca2+ trace (see Fig. 6) or over the last 100 ms of stimulation if shortening was still increasing (see Fig. 7). The criteria for fiber viability were a strong contraction and a rapid increase of fura 2 ratio.

**Statistical Analyses**

Values are means ± SE for the number (n) of muscles tested (force) or fibers/muscles penetrated (action potentials), unless stated otherwise. Statistical analyses involved ANOVA using split-plot designs, with least squares differences used to locate any significant difference. ANOVA was undertaken using the general linear model procedures of SAS statistical software (SAS Institute, Cary, NC). In some cases, paired t-tests were used to examine for differences in control and test responses. Changes are statistically significant (P < 0.05) unless stated otherwise.

**RESULTS**

**Muscle Action Potential Propagation**

Stimulation with transverse wire electrodes by a single pulse that was supramaximal for twitch force triggered an action potential in the surface membrane of soleus fibers on every occasion (244 fibers/27 muscles, setup 1). We tested whether wire stimulation resulted in propagation of an action potential along the surface membrane by changing the distance between the recording microelectrode and the stimulating cathode electrode. This involved moving the microelectrode away from the cathode or reversing the polarity of the stimulating electrodes while the microelectrode stayed at the same location. In both situations, increasing the distance between the microelectrode and the cathode prolonged the delay between the onset of the stimulus artifact and the peak of the action potential. Moving the recording electrode increased the propagation time on average by 1.8 ± 0.4 ms (n = 7). Switching the electrode polarity (Fig. 2) created a greater displacement and increased the propagation time by 3.3 ± 0.7 ms (n = 4).

**Transverse Wire vs. Parallel Plate Electrode Stimulation**

Isometric force was compared in the same muscles when wire stimulation was switched to plate stimulation using standard pulses (20 V, 0.1 ms, setup 2). In soleus muscle (n = 19), the peak force evoked with wires relative to that evoked by plates (bracketing contractions) was 99 ± 1% (not significant) for twitches and 98 ± 1% for tetanic contractions (125 Hz; see Fig. 9A). The relative force for submaximal contractions (10–80 Hz) showed no difference between the two electrode configurations (data not shown). In EDL muscle (n = 5), the peak tetanic force at 200 Hz was slightly lower at 95 ± 1% (see Fig. 9B) and peak twitch force was 98 ± 1% with wire compared with plate stimulation. Soleus muscles were exposed to 30 μM d-tubocurarine for ≥30 min to completely inhibit neuromuscular transmission (11, 20, 33, 39). This treatment resulted in only minor effects on tetanic contractions evoked with plates (i.e., 97 ± 1% of control, n = 8) or wires (i.e., 94 ± 2% of control, n = 8).

**Influence of Stimulation Pulse Parameters on Isometric Force**

Changes of pulse strength or duration have been used to test for changes in sarcolemmal excitability (3, 5–7, 20). We therefore examined the effect of changing pulse parameters on contractile function (setup 2).
Stimulation pulse strength. The twitch force-stimulation strength relationships in soleus and EDL muscles showed small, but significant, differences over the higher submaximal pulse strength range (plate electrodes, 0.1-ms pulse duration). For example, the pulse strength that elicited 75% of maximum twitch force was 2.9 ± 0.1 V (n = 47, SD 1.0 V) for soleus and 3.4 ± 0.4 V (n = 18, SD 1.6 V) for EDL muscle. The pulse strength that first evoked maximum twitch force was 6.1 ± 0.4 V for soleus and 9.3 ± 0.7 V for EDL muscle. Peak tetanic force also reached a plateau when the pulse strength was supramaximal for the twitch in both muscle types (data not shown).

When the twitch force-stimulation strength relationship was determined with wire and plate stimulation in the same five soleus muscles, there was a small rightward shift toward higher pulse strengths with wires (Fig. 3). The pulse strength that elicited 50% of maximum twitch force increased from 1.8 ± 0.1 V with plate stimulation to 3.6 ± 0.5 V with wire stimulation. The subsequent addition of 30 μM d-tubocurarine caused a large rightward shift of this relationship toward higher stimulation strengths for both electrode configurations (Fig. 3). Plate stimulation with 4.4-V pulses normally evoked near-maximum twitch force; in the presence of d-tubocurarine, however, twitches were abolished in four of five muscles, with maximum twitch force generated at ~12 V. With wire stimulation, 8.4-V pulses normally evoked maximum force, and this was reduced to 7% with d-tubocurarine. The pulse strength now had to be increased to 20 V to achieve maximum twitch force.

Stimulation pulse duration. When the pulse duration was increased from 0.1 ms (plate electrodes, 20-V pulse strength), the relative peak twitch force increased significantly to 107 ± 2% (n = 19) at 0.4 ms to 123 ± 5% (n = 8) at 0.7 ms and to 136 ± 3% (n = 32) at 1.0 ms. Peak tetanic force was unchanged over these pulse durations: 102 ± 1% (n = 31, not significant) at 1.0 ms. Greater forces were also evident with 1.0-ms pulses at submaximal frequencies (10–50 Hz, data not shown). In EDL muscle, the peak twitch force increased with 1.0-ms pulses to 129 ± 5% (n = 17), but peak tetanic force was unaffected (i.e., 101 ± 1%, n = 16, not significant).

To confirm whether contractions required the normal Na+–dependent action potential mechanism (3, 37–39, 41), muscles were exposed to 1 μM TTX, which completely blocks action potential generation and T-tubular voltage-dependent Na+ currents (14, 38). In seven soleus muscles, TTX abolished all contractions evoked with 0.1-ms pulses in <5 min. Intriguingly, with increasing pulse duration, a force transient occurred during tetanic stimulation (125 Hz) that became significant with 0.4-ms pulses and reached 19 ± 2% (n = 7) of the control peak tetanic force with 1.0-ms pulses (Fig. 4). In five EDL muscles, TTX prevented the development of tetanic force (200 Hz) evoked with 0.1-ms pulses in 10–15 min. However, tetanic stimulation with 1.0-ms pulses still produced 6 ± 1% (n = 3) of the control tetanic force (Fig. 4). Several features of the contractile responses with 1.0-ms pulses in the presence of TTX differed from that for normal contractions in both muscle types. First, force development in TTX was initially rapid but then declined slowly, i.e., from 25 ± 3% at 2 s to 34 ± 4% at 5 s (n = 3) in soleus muscle, and the late phase of relaxation was prolonged (Fig. 5, inset). Second, the peak force in TTX increased linearly with stimulation frequency, rather than reaching a plateau, over a much higher test frequency range (Fig. 5). Also, the force was greater in soleus than in EDL muscle at each frequency; e.g., the peak force at 300 Hz was 33 ± 6% (n = 4) of control in soleus and 11 ± 2% (n = 3) in EDL muscle. Third, it has been shown that when [K+]o is raised from 4 to 10 mM, the peak tetanic force is reduced (5, 6) because of slow inactivation of many Na+ channels (37). We therefore postulated that if the force generated in TTX also involved Na+ channels, raising [K+]o from 4 to 10 mM should also reduce the force transients. However, the force-frequency

![Fig. 3](image-url)  
Fig. 3. Influence of 30 μM d-tubocurarine on peak twitch force-stimulation strength relationship of soleus muscles subjected to transverse wire or parallel plate stimulation. Values are means ± SE (n = 5). Peak twitch force at each stimulus strength was normalized to that for bracketing twitches evoked with 20-V pulses (0.1-ms duration). Twitches were evoked every 5 s (setup 2). Stimulation strength is voltage delivered to dry electrodes (see DISCUSSION). *Significantly different without and with d-tubocurarine and between control plate and wire responses at ≤4.4 V (indicator is not shown for clarity), P < 0.05 (ANOVA and least significance difference test).

![Fig. 4](image-url)  
Fig. 4. Influence of 1 μM TTX on isometric force during tetanic stimulation with different pulse durations in soleus and extensor digitorum longus (EDL) muscles. Values are means ± SE for soleus (n = 4–7) and EDL (n = 3) muscles. Muscles were stimulated at 125 Hz for 2 s (soleus) and 200 Hz for 2 s (EDL) with 20-V pulses via plate electrodes (setup 2). Peak of force transients at each pulse duration (averaged) in TTX was normalized to maximum tetanic force generated with 1.0-ms pulses immediately before addition of TTX. *Significantly greater than zero, P < 0.05 (ANOVA and least significant difference test).
relationship in TTX was shifted upward at elevated [K\textsuperscript+], as shown at 10 mM K\textsuperscript+ with TTX for soleus muscle (Fig. 5). A similar effect was seen in EDL muscle; e.g., the peak force at 500 Hz was 19±4\% (n=3) at 4 mM K\textsuperscript+ and increased to 29±6\% (n=2) at 11 mM K\textsuperscript+.

**Influence of Stimulation Pulse Parameters on Myoplasmic [Ca\textsuperscript{2+}] and Sarcomere Shortening**

To further explain how longer pulses excite muscle, we measured [Ca\textsuperscript{2+}], (i.e., the fura 2 ratio) during tetanic stimulation in single dissociated FDB fibers from rat (Fig. 6, setup 3). The increase in the fura 2 ratio was greater when the pulse duration was increased from 0.5 to 2.0 ms (5-V pulse strength). For example, in the individual fiber shown in Fig. 6, A and C, 2.0-ms pulses resulted in a 23–28\% greater peak fura 2 ratio over frequencies from 30 to 100 Hz. In all 10 fibers tested, tetanic stimulation (100 Hz) with 2.0-ms pulses increased the peak fura 2 ratio significantly from 1.60 ± 0.17 to 1.98 ± 0.11, although there was no detectable effect in four fibers. Along with the elevated [Ca\textsuperscript{2+}], the extent of sarcomere shortening was also greater. In the representative fiber shown in Fig. 6, B and D, the shortening was 0.05–0.10 \textmu m greater with 2.0-ms than with 0.5-ms pulses. A similar effect was observed in 6 of the 10 fibers tested.

Experiments were also performed on FDB fibers using 200 nM TTX, which prevents action potential generation in the surface membrane of mouse EDL fibers (unpublished observations). TTX eliminated Ca\textsuperscript{2+} release and sarcomere shortening during tetanic stimulation (100 Hz, 0.4 s), when the pulse parameters were 5 V and 0.5 ms, in all fibers tested (Fig. 7). The fura 2 ratio was 0.64 ± 0.03 at rest and 0.63 ± 0.03 during stimulation in the presence of TTX (n=11). In contrast, tetanic stimulation with 2.0-ms pulses in TTX showed Ca\textsuperscript{2+} release in six of these fibers (Fig. 7 A): the fura 2 ratio increased from 0.66 ± 0.04 to 0.89 ± 0.12 (this effect was still significant when tested on all fibers). This small Ca\textsuperscript{2+} response was associated with no shortening (Fig. 7 B) or diminished shortening. The other five fibers showed no change of the fura 2 ratio or shortening in TTX, as seen with the shorter pulses. Moreover, increasing the stimulation pulse strength threefold...
(0.5-ms pulse) in TTX resulted in an increase of the fura 2 ratio: from 0.66 ± 0.05 at 5 V to 1.23 ± 0.28 at 17 V \( (P = 0.08) \). These data confirm that longer or stronger pulses can directly trigger \( \text{Ca}^{2+} \) release in some fibers.

**Influence of Stimulation Pulse Characteristics on Fatigue During Prolonged Tetani**

We use our new understanding about the sites of muscle excitation with different stimulation characteristics to test the contribution of the muscle action potential to the decline of force with prolonged continuous tetanic stimulation, i.e., high-frequency fatigue (HFF).

**Tetanic stimulation with 1.0-ms vs. 0.1-ms pulses.** We postulated that continuous stimulation with longer pulses would slow HFF by reducing action potential failure or by directly activating \( \text{Ca}^{2+} \) release from the SR. Indeed, Fig. 8 confirms that when evoked at 125 Hz the HFF was markedly attenuated throughout the stimulation period with 1.0-ms compared with 0.1-ms pulses in soleus muscle. After 40 s of stimulation, the peak force was 87% of initial force with 1.0-ms pulses and 49% of initial force with 0.1-ms pulses. HFF was also diminished in EDL muscle with 1.0-ms pulses, but only during severe fatigue (Fig. 8). At 20 s of stimulation, the peak force was 32% of initial force with 1.0-ms pulses and 10% of initial force with 0.1-ms pulses. A further observation was a prolonged increase of resting force after stimulation with 1.0-ms pulses. In soleus muscle, this effect amounted to 9 ± 4% \( (n = 7) \) of the peak force at 5 s after stimulation, which then lasted for up to 40 s; the effect was smaller and briefer in EDL muscle, taking up to 10 s.

**Tetanic stimulation with wire vs. plate electrodes.** We hypothesized that a rapid switch from wire to plate stimulation would restore force during severe HFF, if action potential propagation was impaired in the surface membrane during stimulation with wires. In four soleus muscles stimulated at 125 Hz for 35 s with wires, the peak tetanic force was reduced to 37 ± 6% of initial force; then, a switch to plate stimulation rapidly significantly increased force to 69 ± 2%, and at 40 s force remained at 65 ± 2% of initial force (Fig. 9A). In contrast, EDL muscle showed only a small and transient recovery of tetanic force with a switch from wire to plate stimulation (Fig. 9B). In three muscles, wire stimulation at 125 Hz for 15 s caused the peak force to decline to 28 ± 5%; then, with plate stimulation, the tetanic force increased to 35 ± 3% before falling off to 23 ± 3% at 20 s.

**DISCUSSION**

Our main findings are that electrical field stimulation can excite muscle through several different sites, depending on the electrode configuration and pulse parameters. Some stimulation conditions can trigger contraction without generating action potentials. We show that 1) contractions evoked with weaker pulses are prevented with \( \text{D-tubocurarine} \), which blocks neuromuscular transmission (Fig. 3), 2) contractions evoked with moderate pulse parameters are not prevented with \( \text{D-tubocurarine} \) but are abolished with TTX, which blocks voltage-dependent \( \text{Na}^{+} \) channels (Figs. 4 and 7), and 3) contractions and \( \text{Ca}^{2+} \) release evoked with longer or stronger pulses can persist during exposure to TTX (Figs. 4, 5, and 7).
Moreover, the same stimulation characteristics can exert quantitatively different effects in fast- and slow-twitch muscles (Figs. 4, 5, 8, and 9).

**Site of Excitation With Electrical Field Stimulation**

**Electrode configuration.** Contraction evoked with transverse wire or parallel plate electrodes were similar in the control solution when standard (20-V, 0.1-ms) pulses were used (Fig. 9). However, the site of excitation is likely to differ with the type of electrical field generated and the field strength seen by the fibers. First, transverse wire stimulation causes focal excitation of an action potential, which then propagates along the surface membrane (Fig. 2), as seen with other wire electrode arrangements (21, 25, 32, 34). When electrodes are positioned across a muscle (setups 1 and 2), a dipole electrical field exists (Fig. 1C), with the greatest field strength immediately between the electrodes, but declines with distance from the stimulating electrode in the other directions (12, 15). In setup 1, the stimulating electrode is located at one end of the muscle (Fig. 1A), so that an action potential propagates along the entire length of each fiber. In setup 2, the electrodes are placed across opposing sides of the muscle, near the middle (Fig. 1B), so that an action potential propagates in both directions away from the center toward the tendons. Second, with the parallel electrode arrangement (setups 2 and 3), a uniform electrical field exists between the electrodes (Fig. 1D), where the entire muscle is positioned, and the field strength is uniform at a given distance from the stimulating electrode (12, 15). It is thought that plate electrodes trigger action potential simultaneously at all points along the surface membrane, so that propagation is effectively bypassed (32). Consistent with this proposal is the observation that when an action potential can be recorded between parallel electrodes, it occurs so rapidly that the stimulus artifact coincides with the upstroke of the action potential (26). Second, the electrical field strength to which the muscle fibers are exposed depends on the voltage drop at the electrode-solution interface, the electrode separation (or, in reality, the closeness of the fibers to the stimulating electrode), and possible field distortion by the tissue (12). The voltage generated in the bath with the wire and plate electrodes in setup 2 could not be measured because of technical issues and may differ, even though similar pulse parameters were set on the stimulator.

**TTX experiments.** TTX abolished all contractions evoked with 20-V, 0.1-ms pulses in both muscle types (Fig. 4) to implicate involvement of voltage-dependent Na⁺ channels. These data, along with the TTX, confirm that our standard pulses trigger muscle action potentials but do not distinguish between excitation in surface membranes and in T-tubular membranes. Stimulation with even longer pulses resulted in greater forces or shortening of twitch contractions (Fig. 6, B and D), as seen previously for twitches and postulated to be caused by repetitive action potential generation (11, 22, 28, 30). In the present study, we provide evidence for an alternative mechanism by using TTX. Tetanic stimulation with 1.0-ms pulses induced significant force transients during exposure to TTX: 19% of maximum force at 125 Hz in soleus and 6% of maximum force at 200 Hz in EDL muscle (Fig. 4). Similar responses have been reported for mechanically skinned EDL fibers in TTX (35). Such force development is unlikely, inasmuch as TTX failed to block some Na⁺ channels, because contractile properties of the TTX-insensitive force transients were markedly different from normal. 1) The profile of the force transients in TTX was different (Fig. 5, inset). 2) The force-frequency relationship
was different (Fig. 5), with greater forces generated over 300–500 Hz in TTX; yet some stimuli would have arrived during the refractory period for action potentials when Na+ channels are inexcitable (33, 39). 3) Force production was greater at elevated [K+]i (Fig. 5), rather than reduced, as expected due to Na+ channel inactivation (5, 6, 33, 37, 39). These data thus suggest that longer pulses can excite muscle at a site beyond the T-tubular action potential.

Myoplasmic [Ca2+]i measurements. We have shown that potentiation of twitches and submaximal contractions with longer pulses (Fig. 6, B and D) was linked to an increased peak [Ca2+]i (Fig. 6, A and C). Furthermore, longer or stronger pulses could increase [Ca2+]i in the presence of TTX in some fibers, an effect that was sometimes subthreshold for contraction (Fig. 7A). The mechanism for this elevated [Ca2+]i does not require Ca2+ influx across the sarcolemma via Ca2+-dependent slow action potentials (23) or through damaged membrane due to electroporation (15, 16), since tetanic stimulation with 1.0-ms pulses elicits near-maximum force in nominally Ca2+-free solutions (7). We propose that longer or stronger pulses can depolarize the T-tubular membrane to directly activate the DHP receptors and, consequently, trigger Ca2+ release from the SR. This is supported by the observation of potentiated force transients at elevated [K+]i in TTX (Fig. 5), since the T-tubular membrane potential is closer to the threshold for activation of the DHP receptors (10). Moreover, slow-twitch muscle is more sensitive than fast-twitch muscle to direct excitation with long pulses (Figs. 4 and 5), possibly because the DHP receptors require less depolarization to increase force in soleus than in EDL muscle (10) or because Ca2+ uptake by the SR is slower in soleus muscle (9). We cannot establish the precise mechanism for this effect, although it must involve depolarization of the T-tubular membranes (35, 39) by the electrical field or some other mechanism (24) to greater than −50 mV (10).

Physiological excitation. In summary, the most physiological form of artificial stimulation involves excitation via the neuromuscular junction, because all normal peripheral processes remain intact and are involved in the resulting contraction. Also the use of transverse wires is a more physiological form of excitation than parallel plates, since action potentials propagate along the surface membrane, rather than this process being bypassed. Moreover, very strong or long stimulation pulses (especially >0.5 ms) must be avoided in physiological studies, because shorter pulses are adequate to trigger action potentials and longer pulses may excite muscle in a completely nonphysiological way. We now urge that all researchers check the site of excitation in their experiments and provide a full description of the stimulation pulse characteristics and electrode configuration in their research papers.

Implication of Stimulation Characteristics for Understanding Contractile Function Experiments

Twitch force-stimulation strength relationship. The twitch force-stimulation strength relationship has been used as an indirect measure of action potential threshold in muscle fibers (3, 6, 7, 35, 39). Indeed, the response of a single muscle fiber to a single stimulus is the all-or-none generation of an action potential (29, 38, 40, 41), Ca2+ release (9), and twitch force (27). Moreover, individual muscle fibers display a spectrum of action potential thresholds (29, 38, 40, 41), which may account for the gradation of whole muscle force (Fig. 3). d-Tubocurarine caused a rightward shift of the twitch force-stimulation strength relationship (Fig. 3) over the lower pulse strength range (<12 V with plates, <20 V with wires, 0.1-ms duration), which implies that a change over lower pulse strengths may be due to altered neuromuscular transmission or action potential threshold in different muscle fibers.

Electrical stimulation for test contractions. Three such approaches to investigate mechanisms are described. 1) A classical test for deterioration of neuromuscular transmission is used to compare the force produced by nerve stimulation with that produced by muscle stimulation (2, 30). If force is restored, then impairment of neuromuscular transmission is inferred. We suggest that direct stimulation must be strong enough to trigger a muscle action potential (Fig. 3), but without bypassing surface propagation or directly triggering Ca2+ release (Fig. 7). If the latter occurs, a misleading conclusion could be made. 2) Stimulation with stronger/longer pulses has been used to test for impaired excitability in the sarcolemma (3, 5–8, 20). In soleus muscle, increasing the pulse duration to ≤0.25 ms only influences muscle action potentials (Fig. 4), whereas longer (>0.4-ms) pulses can, in addition, directly stimulate Ca2+ release (Figs. 4–7). If force is restored with these longer pulses, the original force reduction can be attributed to reduced excitability, regardless of which site is activated. A related intriguing finding is that 14–20 mM K+ did not completely suppress tetanic force when evoked with 1.0-ms pulses (5, 6). This is most likely to be explained by some nonphysiological Ca2+ release with these long pulses. 3) We have described a new method to test for changes in action potential propagation along the surface membrane, as opposed to the T-tubular membrane, that involves comparison of the force produced by transverse wire stimulation with that produced by parallel plate stimulation (Fig. 9). If tetanic force increases with plate stimulation (surface propagation is bypassed), then T-tubular membranes can still conduct action potentials; hence, failure must have occurred more proximally, i.e., in the surface membrane.

Electrical stimulation tests to understand fatigue during prolonged tetani. Electrical stimulation experiments (Figs. 8 and 9) provided indirect evidence to support the notion that impairment of muscle action potentials cause HFF (4, 13, 20, 25, 26). First, continuous stimulation with the longer 1.0-ms pulses (plate electrodes) prevented ~75% and ~25% of the fatigue in soleus and EDL muscle, respectively (Fig. 8). The force data with 1.0-ms pulses provide evidence for near-maximal Ca2+ release during HFF in soleus muscle; thus the fatigue induced with 0.1-ms pulses is inferred to involve a failure of action potentials to trigger Ca2+ release. Reasons for the smaller protective effect with 1.0-ms pulses in EDL than in soleus muscle may be that pulses cause less direct stimulation of the DHP receptors (Figs. 4 and 5) or another process, such as inactivation of the DHP receptors (10, 39), contributes more to HFF in fast- than in slow-twitch muscle. Second, a rapid switch from wire to plate stimulation during continuous stimulation (Fig. 9A) restored ~50% of the HFF induced with wires in soleus muscle; this implicates a failure of surface propagation when wires are used. The smaller (~10%) restoration of force in EDL muscle on switching from wires to plates (Fig. 9B) further indicates that a distal process, such as...
inactivation of the DHP receptors (10, 39), may have a greater role during HFF in fast- than in slow-twitch muscle. These findings strongly indicate the need for much more consideration of excitability of the surface membrane, rather than just the T-tubular membrane, as a potential site of failure (4, 13, 20, 26, 33, 39). Indeed, changes of action potential pattern have been recorded in the surface membrane during prolonged continuous stimulation in diaphragm muscle (25).

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