Influence of tension time on muscle fiber sarcolemmal injury in rat diaphragm

ERCHENG ZHU, ALAIN S. COMTOIS, LIWEI FANG, NORMAN R. COMTOIS, AND ALEJANDRO E. GRASSINO
Centre de Recherche du Centre Hospitalier de l’Université de Montréal, Montréal, Quebec, Canada H2L 4M1

Zhu, Ercheng, Alain S. Comtois, Liwei Fang, Norman R. Comtois, and Alejandro E. Grassino. Influence of tension time on muscle fiber sarcolemmal injury in rat diaphragm. J. Appl. Physiol. 88: 135–141, 2000.—We hypothesized that the amount of sarcolemmal injury is directly related to the total tension time (TTtot), calculated as mean tension × total stimulation time. Diaphragm strips from Sprague-Dawley rats were superfused at optimal muscle length with Krebs containing procion orange to identify sarcolemmal injury. TTtot was induced by stimulation with 100 Hz for 3 min at duty cycles of 0.02, 0.15, 0.3, and 0.6, or with continuous contractions at 0.2, 0.4, 0.6, and 1.0 of maximal tension. A significant positive correlation between TTtot and the percentage of fibers with injured sarcolemma (r² = 0.63, P < 0.05) is seen. Stimulation (at 100 Hz, duty cycle = 1) resulted in fast fatigue with low injury, likely caused by altered membrane conductivity. Stimulations inducing the largest injury are those showing progressive force loss and high TTtot, where injury may be due to activation of membrane degradative enzymes. The maximal tension measured at 20 min poststimulation was inversely related to the number of fibers injured, suggesting loss of force is caused by cellular injury.

fatigue; isometric contraction; membrane damage; procion; diaphragm muscle

THE MECHANISMS BY WHICH SKELETAL muscles develop injury have been extensively studied in limb muscles, where pliometric contractions were found to be most damaging. Injury has mainly been attributed to high stress and strain, leading to tears in the muscle fibers (12–14). The diaphragm, on the other hand, contracts mainly miometrically and seldom develops pressure >40% of maximal. Our laboratory (19) has previously studied the injury rate of dog diaphragm exposed to mild inspiratory resistance and found that there are injuries that occur relatively more often in the sarcolemma than in the sarcomere. There were no significant injuries while the dogs were breathing at rest (19).

Therefore, whereas the high tension theory as a cause of injury is convincing in eccentric contractions (EC), it does not fully explain the injuries seen in the diaphragm at relatively lower forces. We propose that the amount of injury should be directly related to the level of total tension time (TTtot; the integral of tension developed in the time of loading), a parameter that increases by the level of tension time of contractions.

To test this hypothesis, we measured, in isometric diaphragm strips, the effects of stimulation patterns of both nonfatiguing and fatiguing stimulation. We measured the TTtot developed over a 3-min stimulation protocol, together with the quantity of fibers showing sarcolemmal injury, and the force recovery over 40 min and related them to the amount of injury.

Diaphragm injury may explain the low endurance and prolonged weakness seen after inspiratory resistive loading, which cause fatigue (19).

METHODS

Animal care and preparation. Animal care was conducted under the guidelines of the Canadian Council of Animal Care, with all aspects of the experimental protocols being approved by the institutional animal care and ethics committee. Experiments were performed on mature (250–300 g) male Sprague-Dawley rats from Charles River (St-Constant, Quebec). On their receipt, rats were assigned to square wire-mesh cages in a temperature-controlled (26°C) room with a 12:12-h light-dark cycle. Rats were given free access to standard stock Purina rat chow diets and water.

General procedures. Animals were anesthetized with pentobarbital sodium (50 mg/kg) via an intraperitoneal injection. The entire diaphragm was rapidly excised and immersed in oxygenated Krebs solution for further dissection. Diaphragm strips (3–4 mm wide) were dissected from both right and left sides of the midcostal diaphragm with the fiber insertion at the ribs and the central tendon left intact. The average muscle strip length at optimal muscle length (L₁) was 20.3 ± 1.1 (SE) mm. The average strip weight was 36.7 ± 2.0 mg (wet weight). The strips were mounted in a vertically positioned muscle chamber, with the rib securely clamped to the bottom of the chamber. The central tendon was connected to the force transducer (Kent, Nagashigi, Japan) with no. 4.0 silk thread. The strips were positioned between two sets of platinum electrodes (mounted in the muscle chamber). These electrodes were used to deliver the electrical field stimulation. The strips were continuously superfused (5–10 ml/min at 35°C) with fresh Krebs solution, which had a composition of (in mM) 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1 KH₂PO₄, 25 NaHCO₃, and 11.0 glucose. The Krebs solution was equilibrated with 95% O₂-5% CO₂ to a pH of 7.35 and was bubbled with this gas mixture throughout the experiment. The fluorescent molecular tracer procion orange 14 (Sigma Chemical) was added to the Krebs solution (0.15% wt/vol) for

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were adjusted to the identification of fibers that undergo sarcolemmal disruption. The total exposure time of the strips to the bath solution was 90 min in all studies. After an equilibration period of 15 min, diaphragm strips were adjusted to \( L_o \), where the \( L_o \) was defined as the length at which peak isometric tetanic tension was produced. The muscle strips were stimulated (model 548 stimulator, Grass, Quincy, MA) to achieve maximum tetanic force, where the final voltage (−6 V) was set at 1.3 times the voltage that gave maximal force; the stimulation frequency was 100 Hz, and the train duration was set at 600 ms (the train duration of a single rectangular current pulse was 0.3 ms). After the 3-min stimulation with the muscle recovering, contractility was measured by applying 600-ms tetanic stimulations, which were contractions given at 100-s intervals for 40 min until the recovery period was completed. The force signals produced by tetanic contractions were digitized (DT2821; Data Translation, Marlboro, MA) at a sampling rate of 1,000 Hz and fed into a personal computer for later analysis. The force was expressed in newtons per square centimeter, and the muscle cross-sectional area was calculated as the ratio of trimmed muscle mass (g) to strip length (cm) \( \times 1.056 \, \text{g/cm}^3 \) (muscle density) \( (6) \). The temperature of the bath was maintained at 35°C for the duration of the experiment.

Experimental protocols. In protocol 1, the diaphragm strips \( (n = 37) \) were subjected to repeated 100-Hz maximal isometric tetanic contractions for 3 min at the following initial duty cycles (DCs): 0.02, 0.15, 0.3, and 0.6. DCs were changed by adjusting the rest-time interval. In protocol 2, four groups of muscle strips were stimulated continuously for 3 min. The sustained isometric contractions were initiated at either 20, 40, 60, or 100% of maximal tension by adjusting the stimulation frequency between 6 and 100 Hz. Table 1 lists the number of diaphragm strips in each group, the stimulation frequency, the tension time index (TTI) \( \left( \text{tension/tension}_{\text{max}} \times C/(C + R) \right) \), where tension \(_{\text{max}} \) is maximum tension, C is contraction time, and R is rest time, as a unitless parameter to describe the stimulation pattern at the start of each protocol. DC, relative force, TTI \(_{\text{st}} \), fraction of injured cells, and the number of stimulation trains per 3 min for each protocol. In addition, 19 paired strips were set at \( L_o \) in the muscle chamber without stimulation and served as control for cell membrane injury counting.

The digitized tetanic contractions generated during both the 3-min contractile protocol and the 40-min recovery period were all analyzed for the following parameters as illustrated in Fig. 1: the peak tetanic force (PF), defined as the maximum force developed during a contraction; the PF\(_{\text{st}} \), defined as the PF obtained at the start of the 3-min stimulation protocol; the PF\(_{\text{end}} \), defined as the PF at the end of the 3-min stimulation protocol; both the maximum contraction and relaxation rate \( (dF/dt \, \text{and } -dF/dt, \text{respectively}) \), the muscle contractile prop-

### Table 1. Parameters of stimulation protocols and results of percentage of fibers injured

<table>
<thead>
<tr>
<th>Stimulation Frequency, Hz</th>
<th>n</th>
<th>TTI (_{\text{st}} )</th>
<th>C/(C + R)</th>
<th>( P_{\text{PF}} ) N/cm²</th>
<th>F/F(_{\text{PF}} ) max, %</th>
<th>No. of Trains</th>
<th>n/3 min</th>
<th>Total Stimulation Time, s</th>
<th>TT(_{\text{st}} )</th>
<th>N.cm⁻².s⁻¹</th>
<th>%Injured</th>
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<td><strong>Protocol 1</strong></td>
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<tr>
<td>100</td>
<td>7</td>
<td>0.02</td>
<td>600/30,000</td>
<td>23.5 ± 2.1</td>
<td>100</td>
<td>6</td>
<td>3.6</td>
<td>126 ± 13</td>
<td>2.7 ± 0.5</td>
<td>100</td>
<td>100</td>
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<tr>
<td>100</td>
<td>7</td>
<td>0.15</td>
<td>600/4,000</td>
<td>22.0 ± 1.8</td>
<td>100</td>
<td>45</td>
<td>27.0</td>
<td>564 ± 35</td>
<td>13.7 ± 2.3</td>
<td>100</td>
<td>100</td>
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<tr>
<td>100</td>
<td>15</td>
<td>0.3</td>
<td>600/2,000</td>
<td>20.8 ± 1.0</td>
<td>100</td>
<td>90</td>
<td>54.0</td>
<td>585 ± 40</td>
<td>25.8 ± 2.6</td>
<td>100</td>
<td>100</td>
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<tr>
<td>100</td>
<td>8</td>
<td>0.6</td>
<td>600/1,000</td>
<td>22.7 ± 1.2</td>
<td>100</td>
<td>180</td>
<td>108.0</td>
<td>727 ± 37</td>
<td>16.7 ± 4.7</td>
<td>100</td>
<td>100</td>
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<td><strong>Protocol 2</strong></td>
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<td>6–10</td>
<td>5</td>
<td>0.2</td>
<td>1,000/1,000</td>
<td>22.4 ± 1.6</td>
<td>20</td>
<td>1</td>
<td>180.0</td>
<td>569 ± 56</td>
<td>8.7 ± 3.4</td>
<td>100</td>
<td>100</td>
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<tr>
<td>10–20</td>
<td>6</td>
<td>0.4</td>
<td>1,000/1,000</td>
<td>23.2 ± 1.5</td>
<td>40</td>
<td>1</td>
<td>180.0</td>
<td>658 ± 70</td>
<td>21.4 ± 2.2</td>
<td>100</td>
<td>100</td>
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<tr>
<td>20–30</td>
<td>6</td>
<td>0.6</td>
<td>1,000/1,000</td>
<td>21.9 ± 1.5</td>
<td>60</td>
<td>1</td>
<td>180.0</td>
<td>883 ± 39</td>
<td>18.9 ± 5.9</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>1.0</td>
<td>1,000/1,000</td>
<td>23.7 ± 1.1</td>
<td>100</td>
<td>1</td>
<td>180.0</td>
<td>424 ± 28</td>
<td>10.2 ± 1.9</td>
<td>100</td>
<td>100</td>
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</table>

Values are means ± SE where shown; n, no. of strips. Note: TTI\(_{\text{st}} \), tension time index at start of each protocol, where TTI = tension/tension\(_{\text{max}} \) \times contraction time (C)/(C + rest time (R)); \( P_{\text{PF}} \), maximal tension at optimal muscle length; F/F\(_{\text{PF}} \) max, percentage of maximal force induced. No. of trains, no. of stimulation trains performed within the 3-min stimulation protocol. Total stimulation time, sum of stimulation time. TTI\(_{\text{st}} \) is same as in protocol 2. TT\(_{\text{st}} \) total tension time during 3-min stimulation period. TT\(_{\text{st}} \) = \( \Sigma F \times dF \).

%Injured, fraction of diaphragm fibers showing procion in cytoplasm expressed as percentage of total fibers counted.

![Fig. 1. Top trace: A 600-ms-long tetanic 100-Hz supramaximal electrical stimulation during protocol 1. PF\(_{\text{st}} \) (solid line), value of peak force (PF) expressed in N/cm² at start of 3-min stimulation protocol. F\(_{\text{600st}} \), percentage of force generated at 600 ms within contraction to maximal force at start of poststimulation period. F\(_{\text{600end}} \), force of end of tetani, expressed as percentage of PF. Dotted line represents last tetani at end of 3rd min of stimulation (PF\(_{\text{end}} \)). Bottom traces: maximal contraction rate (dF/dt) and maximal relaxation rate (−dF/dt) at both start (st) and end. These parameters were measured constantly throughout 3-min stimulation period and 40-min recovery period.](image)
RESULTS

Two protocols were carried out. In protocol 1, stimulation was done with supramaximal voltage at 100 Hz. The DC was varied to generate initial TTI values of 0.02–0.6. In protocol 2, the DC was constant at 1 (continuous contraction). The initial force was varied by changing the frequency of stimulation. Parameters defining the stimulation patterns are shown in Fig. 1.

Figure 2A shows the time course of force obtained from protocol 1 during 3 min of stimulation. Figure 2B shows the force elicited every 100 s, via a 600-ms electrical stimulation given over the 40-min recovery period. The rate of force decay increases in direct relationship to the initial TTI. The lowest TTI, 0.02, shows no force loss, fatigue becomes evident at all the other initial TTI values. The rate of decay of force was quantified as TD50 (the time of decrease of force to 50% of maximal force), and values are shown in Table 2. Time course of PF recovery is shown in Fig. 2B. Notice that the TTI 0.02, a nonfatiguing protocol, shows the same PF at the start and the end of the run (PFst and PFend, respectively). The PF20, or force measured after 20 min poststimulation, was ~93% of control. At fatiguing protocols, force decreased the most at TTI 0.6 and 1.0 with levels ~88%. Force recovery from fatigue runs was incomplete. The highest recovery in force was seen at TTI 1.0 (74%), and the lowest was at TTI 0.3–0.6 (reaching 55–60% of control and deteriorating further with time). Similar parameters measured in protocol 2 are shown in Fig. 2, C and D. Values of parameters related to muscle contractility are shown in Table 2.

Fig. 2. Protocol 1. A: PF (expressed as percentage of maximal) of intermittent stimulation trains along 3-min stimulation for all runs. TD50, time at which force fell to 50% of maximum. B: time course of PF measured every 100 s during recovery for all runs. PF20, PF value at 20 min into recovery. Different symbols show initial tension time of each run (tension time index [TTI]). Protocol 2. C: time course of PF during sustained 3-min contraction runs. TD50, time at 50% drop of initial force. Notice runs were done at submaximal levels of stimulation and in a continuous contraction (duty cycle = 1). D: recovery time of PF. Symbols indicate various initial TTI for protocol 2. Values are means ± SE.

dtions, which were measured from the dF/dt of tetanic contrac-
tions (9); and the tension at 600 ms relative to the peak
tetanic tension within the contraction. Figure 2 shows the
time course of PF during the 3-min stimulation period (A) and
the recovery period (B) for protocol 1. The TD50 is the time to
reach 50% decay from the PF at the start of the stimulation
protocol. The PF20 is measured at 20 min into the recovery
period. Figure 2, C and D, shows data from protocol 2, indicating the TD50 and PF20 values. Both protocols were
tested up to 40 min after the 3-min stimulation period.

Quantification of fiber sarcolemmal injury. Immediately
after the recovery period was completed, the muscle strips
were rinsed in normal Krebs solution for 5 min, quick-frozen
in isopentane precooled with liquid nitrogen, and preserved
at −80°C. Serial sections (10 µm thick) were cut in the
transverse plane with a cryostat microtome (Leica Cryocut
1800, Heidelberg, Germany). To assess the amount of sarco-
lemmal injury, transverse frozen sections of rat diaphragm
were randomly selected and photographed by using epifluo-
rescence microscopy (Carl Zeiss D-7082, Oberkochen, Germany). Approximately 10–15 microscopic fields per muscle were analyzed by using the fluorescence filter setting at a magnification level of ×100. Muscle fibers that demonstrated a clear increase in cytoplasmic fluorescence (i.e., fibers containing the procion orange tracer dye) were counted, and the percent-
age of dye-positive fibers on each diaphragm section was
determined. Figure 3 shows samples of muscle exposed to
different TTI. The areas with sectioning artifacts (folds, tears,
etc.) were excluded, as were the edges of the sections to avoid
areas damaged by the muscle dissection. A minimum of 1,000
diaphragm fibers was counted in each muscle.

The counting was done after the double-blind method. The
slides were coded by one person (A. E. Grassino), who did not
take part in the reading. All slides were quantified to indicate
the percentage of fibers presenting sarcolemmal injury by one
observer (E. Zhu) and then by a second observer (A. S.
Comtois) with knowledge of the experimental condition.
The code was broken after all morphological assessments had
been completed. The result of injured fibers is expressed as
percentage of positive fibers to total fibers counted. The
variation in the percentage of fibers in terms of interobserver
correlation (between observers E. Zhu and A. S. Comtois) was
r2 = 0.9762 (P < 0.01) and in terms of the intraobserver
variation (2 measurements made by the same observer) was
r2 = 0.9824 (P < 0.01).

Statistical analysis. Results are expressed as mean values
± SE, unless otherwise indicated. Differences between

groups were assessed by one-way ANOVA with post hoc
application of the Student-Newman-Keuls test. Statistical
significance was set at P < 0.05, unless otherwise specified.
Fig. 3. Cross sections of rat diaphragms showing sarcolemmal injury at various initial TTI values: 0.02 (a), 0.15 (b), 0.3 (c), and 0.6 (d). Cells were filled with procion (showing in white). Fibers with intact membranes are dark. Procion in interstitial space delineates limits of fibers and areas of interstitial tissue. Counting for quantification included 10–15 microscopic fields (magnification ×100) and included counting over 1,000 fibers to derive percentage of fiber injury on a given condition. *Example of injured fibers (positive fibers); **example of noninjured fibers (negative fibers); arrow points to typical sample area that was excluded in quantification of injured fibers for all samples analyzed.

Table 2. Muscle contractility during and after various stimulation protocols

<table>
<thead>
<tr>
<th>TTI</th>
<th>$PF_{st}$, N/cm²</th>
<th>$PF_{end}$, N/cm²</th>
<th>$PF_{20}$, N/cm²</th>
<th>$dF/dt_{st}$, N·cm⁻²·s⁻¹</th>
<th>$dF/dt_{end}$, N·cm⁻²·s⁻¹</th>
<th>$dF/dt_{20}$, N·cm⁻²·s⁻¹</th>
<th>TD₅₀, min</th>
<th>F₆₀₀₀st, %</th>
<th>F₆₀₀₀end, %</th>
<th>F₆₀₀₀₁₀₀s, %</th>
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<tr>
<td>0.02</td>
<td>23.5 ± 2.1</td>
<td>22.9 ± 2.3</td>
<td>21.2 ± 2.3</td>
<td>377 ± 25</td>
<td>370 ± 20</td>
<td>432 ± 38</td>
<td>509 ± 87</td>
<td>97.7 ± 0.9</td>
<td>97.4 ± 0.9</td>
<td>93.0 ± 0.5</td>
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<tr>
<td>0.15</td>
<td>22.0 ± 1.8</td>
<td>7.1 ± 0.6</td>
<td>14.5 ± 1.2</td>
<td>439 ± 64</td>
<td>147 ± 19</td>
<td>682 ± 68</td>
<td>506 ± 31</td>
<td>94.9 ± 4.1</td>
<td>94.0 ± 1.5</td>
<td>95.9 ± 1.1</td>
</tr>
<tr>
<td>0.3</td>
<td>20.8 ± 1.0</td>
<td>2.3 ± 0.3</td>
<td>11.9 ± 0.9</td>
<td>459 ± 29</td>
<td>90 ± 7</td>
<td>707 ± 33</td>
<td>356 ± 24</td>
<td>97.9 ± 0.7</td>
<td>68.7 ± 7.4</td>
<td>94.4 ± 1.1</td>
</tr>
<tr>
<td>0.6</td>
<td>22.7 ± 1.2</td>
<td>1.9 ± 0.3</td>
<td>16.1 ± 1.3</td>
<td>399 ± 54</td>
<td>86 ± 9</td>
<td>682 ± 140</td>
<td>17 ± 2</td>
<td>96.6 ± 1.0</td>
<td>55.7 ± 8.2</td>
<td>96.3 ± 1.6</td>
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<tr>
<th>Protocol 2</th>
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<tr>
<td>0.2</td>
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<tr>
<td>0.4</td>
</tr>
<tr>
<td>0.6</td>
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<td>1.0</td>
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All values are means ± SE. TTI, total tension time; $PF_{st}$, peak force of first contraction at start of 3-min stimulation period; $PF_{end}$, peak force of last contraction of stimulation period; $PF_{20}$, peak force at 20 min of recovery; $dF/dt_{st}$ and $dF/dt_{end}$, maximal contraction rate at start and end of stimulation period, respectively; $dF/dt_{20}$, maximal relaxation tension rate at start and end of stimulation period, respectively; $dF/dt_{20}$, maximal relaxation tension rate at 20 min of recovery from stimulation protocols; TD₅₀, time to reach 50% decrease in force from start of stimulation. F₆₀₀₀st, F₆₀₀₀end, and F₆₀₀₀₁₀₀s, percentage of force generated at 600 ms within a contraction to maximal force at start or end of stimulation protocol or at 100 s poststimulation period, respectively. Results are taken from each of the stimulation trials.
The percentage of fibers found to have procion in the cytoplasm is expressed as a percentage of total fibers counted for each protocol; values are included in Table 1. Samples of sarcolemmal injury are illustrated in Fig. 3.

The rate of rise of force at the start of the 3-min stimulation period and at the end \((\frac{dF}{dt})_{\text{end}}\) is an indication of the contractile mechanism deterioration. It was not affected in the nonfatigue run. The largest decrease in the rate of rise of force occur at TTI 0.3–0.6 (19–27%).

The \(-\frac{dF}{dt}\) is an expression of cytosolic \(Ca^{2+}\) management. A slow relaxation rate is seen when there is free \(Ca^{2+}\) accumulation intracellularly (3). There was no change between \(\frac{dF}{dt}\) at the start of the stimulation period and \(\frac{dF}{dt}_{\text{end}}\) in the nonfatigue run. The highest decay is seen at initial TTI 0.6 in protocol 1 and at initial TTI 1.0 in protocol 2, with a decrease to 2–5% of \(-\frac{dF}{dt}\) at the start of the stimulation period \((-\frac{dF}{dt}_{\text{st}})\).

The loss in \(-\frac{dF}{dt}\) is a function of the intensity of the stimulation pattern. \(-\frac{dF}{dt}_{20}\) expresses the recovery of relaxation rate after 20 min of rest. Compared with \(-\frac{dF}{dt}_{\text{st}}\), we note a decreased value of \(-\frac{dF}{dt}_{20}\) in all runs except the nonfatiguing run (TTI 0.02). On the other hand, \(-\frac{dF}{dt}_{20}\) values are considerably higher than \(-\frac{dF}{dt}\) at the end of the stimulation period \((-\frac{dF}{dt}_{\text{end}}\) of the stimulation run, showing some recovery. The run at TTI 0.6 recovered partially, with \(~50\%\) of \(-\frac{dF}{dt}_{\text{st}}\). Figure 4 shows the \(-\frac{dF}{dt}_{\text{end}}\) and \(-\frac{dF}{dt}_{\text{st}}\), demonstrating a similar loss in both.

**DISCUSSION**

The objective of this study was to establish if there was a relationship between the amount of injury and \(TT_{\text{tot}}\). Our major findings indicate the following. 1) The \(TT_{\text{tot}}\) developed by the muscle shows a direct relationship to the amount of sarcolemmal injury, as indicated in Fig. 5A. 2) The capacity to recover force after fatigue is inversely proportional to the number of injured fibers (Fig. 5B). 3) Neither the maximal level of tension developed nor the DC alone was related to the amount of injury.

Critique of methods to measure sarcolemmal injury. Many techniques have been used to assess the magnitude of sarcolemmal injury. Injecting procion orange into the blood circulation in vivo or adding it to the muscle bath are simple and highly sensitive techniques for the detection of sarcolemmal gaps in the diaphragm fibers (19). Procion orange 14, a fluorescent compound with a small molecular weight, is impermeable in healthy muscle membranes; it enters only into the cytoplasm when there is a membrane gap. The advantage of the procion orange technique for in vitro studies of the diaphragm is that the muscle is both flat and thin (between 15 and 25 cells across) and thus allows a good diffusion of the Krebs solution to the central fibers and creates a clear image of the distribution of fibers with sarcolemmal injury. Other techniques, such as measuring the amount of enzymes released from the cytoplasm, such as myoglobin or enzyme efflux [e.g., creatine kinase and lactate dehydrogenase (18) or antiserum albumin (11)], have all been used with success in both in vivo and in vitro skeletal and cardiac muscle studies. In our experiments, however, the total amount of enzyme or protein release from the cyto-

![Fig. 4. Correlation between \(dF/dt\) and \(-\frac{dF}{dt}\) at end of stimulation runs (\(dF/dt_{\text{end}}\) and \(-\frac{dF}{dt}_{\text{end}}\), respectively). ○, Protocol 2; ●, protocol 1.](image)

![Fig. 5. Relative amount of muscle fibers showing sarcolemmal injury in relation to total tension time \((TT_{\text{tot}}; \text{A})\) and \(PF_{20} (\text{B})\). A: there was significant change in sarcolemmal injury with \(TT_{\text{tot}}\). B: capacity to recover force after fatigue at 20 min from stimulation protocols is inversely proportional to no. of injured fibers. *Amount of fibers injured in control group without stimulation \((0 TT_{\text{tot}}). \text{○, Protocol 2; ●, protocol 1.}](image)
plasm would be relatively small, because the muscle mass is 36.7 ± 2.0 mg and because the enzyme or protein efflux would dilute very rapidly in the 50-mL muscle bath. Furthermore, the flow rate of the Krebs solution in the bath is large at 5–10 mL/min. Consequently, whatever the amount of enzymes or proteins released by injured muscle fibers, it would quickly become diluted and would rapidly be removed from the bath. The sample taken from the bath would probably underestimate and vary considerably in the actual measurement of damage. Therefore, we believe that, for studies of the diaphragm in a muscle chamber preparation, the fluorescent dye (procion orange) technique is more sensitive than measuring the release of enzymes or proteins into the muscle bath.

Use of TTI and TTtot to evaluate muscle injury. We use the term TTI to define the pattern of stimulation in terms of tension/tension_max × DC (unitless). The term TTtot is defined as the time integral of tension throughout the 3-min protocol. How do TTI and TTtot relate? In nonfatiguing contractions, they are linearly related. In fatiguing contractions, the correlation is shown in Fig. 6, suggesting a deterministic bimodal relationship. A progressive increase in TTI up to ~0.4–0.6 results in a linear increase in TTtot, with the peak being reached at TTI of ~0.6. Beyond 0.6, TTtot decreases. In the ascending leg (TTI from 0.15 to 0.4), the increase in TTtot correlates directly to the increase in sarcolemmal injury. This may be caused via a cytosol Ca^{2+} increase, activating Ca^{2+}-sensitive degradative pathways, which leads to SR and membrane lesions. Stimulation at TTI of 1.0 results in a rapid fatigue, a low value in TTtot, and a lower level of injury. It describes a parabolic relationship. The decrease in TTtot at TTI of 0.6–1.0 may be due to the development of high-frequency fatigue affecting mainly the membrane depolarization, thereby causing loss of tension, which protects the integrity of cell metabolism and structure. TTI 0.02 is a nonfatigue pattern, and little injury is expected.

The muscle strips submitted to fatiguing or nonfatiguing intermittent stimulations in protocol 1 develop maximal tension during maximal stimulation at the start of the run. The highest relative number of injured fibers was found at TTI 0.3 and 0.4, yielding the maximal TTtot. The fatigue threshold of TTI causing fatigue in vivo is reported at ~0.15 (4), a level at which, in this study, we found 15% of fibers showing sarcolemmal injury. Human subjects can sustain a breathing pattern of TTI 0.15 for slightly longer than 1 h before respiratory muscle fatigue sets in and leads to task failure. There are, however, little data available to clarify the relationship between fatigue and injury (4).

Diaphragms of in vivo dogs breathing at TTI 0.12 did show a 7% fiber injury (19). In the present preparation, TTI of 0.15 and 0.2 resulted in fatigue and injury in the 8.7–13.7% range. In protocol 2, during sustained isometric stimulation with varied frequency of stimulation, the highest relative number of fibers showing sarcolemmal injury (~21%) was found at a TTI of 0.4 and not at TTI 1.0 where the maximal force was generated. These results suggest that a rapid fatigue, induced by failure of the membrane electrical potential to propagate beyond T tubes, appears to serve a protective role in muscle sarcolemmal injury. This behavior also indicates that force developed by the muscle during contractions is not the only factor affecting sarcolemmal injury. Other influencing factors, such as the DC, local temperature, and the production of oxidants, can play a role in causing injury.

Proposed mechanisms of sarcolemmal injury. Most of the hypotheses that support “mechanical stress” as a cause of muscle injury during exercise are based on studies of EC. EC indeed produce higher tension per cross-sectional area than do concentric contractions. It was proposed that EC stress all of the structural elements of the muscles, which are in series, and, eventually, pull apart the sarcomere with disruption of the A and Z bands (2), as well as cause disruption of the plasma membrane and sarcolemma by shearing forces (14). Also, the amount of sarcolemmal injury seen with pliometric contractions has been found to be directly correlated with the magnitude of mechanical stress in the mdx mouse, a strain lacking the dystrophin gene (15). Thus these observations support the hypothesis that cytoskeletal proteins that link the sarcolemma to contractile proteins are involved (17). In isometric contractions, there is evidence that the total free Ca^{2+} concentration is elevated by exercise. A high Ca^{2+} level in the cytosol is related to fiber injury (7) as well as to the protein release and cytosolic enzyme efflux (10). The elevation of cytosol Ca^{2+} can be secondary to disruption of SR membrane or to extracellular influx of Ca^{2+} (14).

In our work, the time at which the muscle strips lost 50% of their maximal force (TD_{50}) is strongly related to the stimulation TTI, as shown in Table 2. The lowest initial TTI (0.02) did not produce fatigue and also

![Fig. 6. Relationship between TTI and TTtot. A progressive increase in initial TTI up to ~0.6 seems to result in a linear increase in TTtot. Beyond 0.6, TTtot decreases until initial TTI of 1.0. Polynomial regression is TTtot = 131 + (2.207 × TTI) − (1.827 × TTI^2) − (81 × TTI^3). R^2 = 0.90, P < 0.05. ○, Protocol 2; ●, protocol 1.](image-url)
showed a low TT\textsubscript{tot} (Table 1). The highest TTI (1.0) produced a precipitous decrease in force (TD\textsubscript{50} of 12 s), and, therefore, TT\textsubscript{tot} also remains low.

This fatigue can be defined as high frequency and occurs at the T tubular level because of a moderate and fully reversible state of action potential propagation failure, which is supported by the changes in the values of the percentage of force generated at 600 ms within a contraction to maximal force (13) (Table 2). Here, the cell’s physical integrity is not affected, because the failure to contract occurs at the excitation phase rather than at the sarcomere level. The runs with intermediary levels of TTI result in higher TT\textsubscript{tot} and a higher amount of injury. In the runs with intermediary levels of TTI and maximal TT\textsubscript{tot}, the fatigue is likely to be originated at the contractile level. For example, the −dF/dt at the end of the 3-min run (−dF/dt\textsubscript{end}, Table 2) decreases considerably and shows an inverse relation with the TT\textsubscript{tot} developed. The −dF/dt decreases significantly in runs having a high percentage of injured fibers.

Other mechanisms have been shown to participate in the process of sarcolemmal injury. For example, it has been reported that oxygen free radicals are formed in the diaphragm during inspiratory resistive breathing, and these radicals cause impaired diaphragm function (1). Recent reports support the view that the increase in oxygen free radicals influences Ca\textsuperscript{2+} movements in the cell by modifying the proteins associated with the Ca\textsuperscript{2+} release process (5, 11a).

In real life, the diaphragm is designed to shorten, rather than generate, high force. In fact, shortenings of 20% from L\textsubscript{0} are common during hyperpnea, changes in body position, or continuous positive airway pressure. We suspect length is a relevant factor in diaphragm injury. This aspect was not explored in this work, where length was constant (8, 16).

In conclusion, the integrity of skeletal muscle fiber membrane is essential for muscle fiber homeostasis and for the performance of muscle tension. Our results indicate that the amplitude of force generation, both during the fatiguing protocol and in the subsequent recovery period, was greatly affected by the initial tension time protocol of stimulation applied during a 3-min period. The TT\textsubscript{tot} developed was directly related to the number of muscle fibers having sarcolemmal injury. The force recovery after 20 min of rest is inversely related to the amount of fiber injury. This is in agreement with previous results reported by West-Jordan and colleagues (18), who showed that the maximal creatine kinase efflux is inversely related to the force recovery after poststimulation. We found injury at effort levels in the range of those causing fatigue. Fiber injury may be a cause of prolonged force loss after strong efforts.

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Address for reprint requests and other correspondence: A. E. Grassino, Centre de Recherche du CHUM, Campus Notre-Dame, 1560 Rue Sherbrooke Est., Porte I-2158, Montreal, Quebec, Canada H2L 4M1 (E-mail: agrassino@hotmail.com). Received 11 june 1998; accepted in final form 31 August 1999.

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