Role of extracellular [Ca\(^{2+}\)] in fatigue of isolated mammalian skeletal muscle

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Department of Physiology, School of Medicine, University of Auckland, Auckland; Kinesiology Group, School of Physical Education, University of Otago, Dunedin; Auckland School of Physiotherapy, Auckland Institute of Technology, Auckland; and Department of Physiology, School of Medicine, University of Otago, Dunedin, New Zealand

Cairns, Simeon P., Wayne A. Hing, John R. Slack, Roland G. Mills, and Denis S. Loiselle. Role of extracellular [Ca\(^{2+}\)] in fatigue of isolated mammalian skeletal muscle. J. Appl. Physiol. 84(4): 1395–1406, 1998.—The possible role of altered extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) in skeletal muscle fatigue was tested on isolated slow-twitch soleus and fast-twitch extensor digitorum longus muscles of the mouse. The following findings were made. 1) A change from the control solution (1.3 mM [Ca\(^{2+}\)]\(_o\)) to 10 mM [Ca\(^{2+}\)]\(_o\), or to nominally Ca\(^{2+}\)-free solutions, had little effect on tetanic force in nonfatigued muscle. 2) Almost complete restoration of tetanic force was induced by 10 mM [Ca\(^{2+}\)]\(_o\) in severely K\(^{-}\)-depressed muscle (extracellular K\(^{-}\) concentration of 10–12 mM). This effect was attributed to a 5-mV reversal of the K\(^{-}\)-induced depolarization and subsequent restoration of ability to generate action potentials (inferred by using the twitch force-stimulation strength relationship). 3) Tetanic force depressed by lowered extracellular Na\(^{+}\) concentration (40 mM) was further reduced with 10 mM [Ca\(^{2+}\)]\(_o\). 4) Tetanic force loss at elevated extracellular K\(^{-}\) concentration (8 mM) and lowered extracellular Na\(^{+}\) concentration (100 mM) was partially reversed with 10 mM [Ca\(^{2+}\)]\(_o\), or markedly exacerbated with low [Ca\(^{2+}\)]\(_o\). 5) Fatigue induced by using repeated tetani in soleus was attenuated at 10 mM [Ca\(^{2+}\)]\(_o\) (due to increased resting and evoked forces) and exacerbated at low [Ca\(^{2+}\)]\(_o\). These combined results suggest, first, that raised [Ca\(^{2+}\)]\(_o\) protects against fatigue rather than inducing it and, second, that a considerable depletion of [Ca\(^{2+}\)]\(_o\) in the transverse tubules may contribute to fatigue.

Changes in Extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) have been proposed to contribute to skeletal muscle fatigue, that is, a reduced muscle force production (for reviews see Refs. 14, 15, 29). The small diameter of the transverse (t)-tubular lumen and the long diffusion path between it and the bulk extracellular fluid, together with the presence of both voltage-activated Ca\(^{2+}\) channels and Ca\(^{2+}\)-ATPase proteins in t-tubular membranes (2, 25, 29), implicate the t tubule as the most important site at which activity-dependent changes in [Ca\(^{2+}\)]\(_o\) may occur to influence force production. In fact, much indirect evidence from isolated muscle preparations suggests that changes in t-tubular [Ca\(^{2+}\)] may occur during repetitive activation (1, 3–5, 19, 22, 26).

An influx of Ca\(^{2+}\) from the t-tubular lumen to the myoplasm through voltage-activated Ca\(^{2+}\) channels takes place during repetitive stimulation (1, 2, 28). This influx, combined with slow Ca\(^{2+}\) diffusion from the bulk extracellular solution into the t-tubular lumen (1), would be expected to result in a substantial lowering of [Ca\(^{2+}\)]\(_o\) in the depths of the t tubules (1, 14, 15, 26, 29). One study, using \(^{40}\)Ca\(^{2+}\), indicates that t-tubular [Ca\(^{2+}\)]\(_o\) is reduced during low-frequency fatigue (22). In addition, t-tubular Ca\(^{2+}\) may become depleted during prolonged depolarization (1, 26), a condition known to occur in some types of fatigue (14, 23, 29, 31). Indeed, in a recent abstract (18), work in which a low-affinity Ca\(^{2+}\) indicator was used has confirmed that t-tubular [Ca\(^{2+}\)]\(_o\) is lowered during voltage-clamp depolarization in frog muscle, although this did not occur with the fatigue protocols the authors adopted.

There is also evidence that [Ca\(^{2+}\)]\(_o\) becomes elevated during fatigue. Data from \(^{40}\)Ca\(^{2+}\) studies have revealed an enhanced Ca\(^{2+}\) efflux into the t-tubular system during fatigu ing twitch stimulation of frog muscle (3–5) and during repeated tetanic stimulation of rat diaphragm muscle (22). Similar conclusions have also been drawn from changes in birefringence following continuous tetanic stimulation (19). An enhanced Ca\(^{2+}\) efflux into the t tubules may arise from greater Ca\(^{2+}\) extrusion via increased Ca\(^{2+}\)-ATPase activity in t-tubular membranes (3). Changes of t-tubular [Ca\(^{2+}\)]\(_o\), during fatigue are, therefore, likely to reflect a balance between changes in Ca\(^{2+}\)-ATPase-mediated extrusion and influx through voltage-activated Ca\(^{2+}\) channels.

Evidence for the influence of altered [Ca\(^{2+}\)]\(_o\) on fatigue from contractile studies is also conflicting. It has repeatedly been shown that nominally Ca\(^{2+}\)-free solutions have little effect on force in isolated nonfatigued muscle (11, 25, 32). Other investigations have shown that an increase in [Ca\(^{2+}\)]\(_o\) from 2 to 5–20 mM depressed peak force by up to 40% in isolated nonfatigued frog muscle (20, 21), which provides support for the notion that raised t-tubular [Ca\(^{2+}\)]\(_o\) may contribute to fatigue. This depressive effect of elevated [Ca\(^{2+}\)]\(_o\) was attributed to impaired conduction of action potentials in the t tubules (19–21). Similarly, the etiology of some, but not all, types of fatigue has been localized to action potential failure in the t tubules (7, 13, 14, 31). Despite these findings, it has been argued from other evidence that raised [Ca\(^{2+}\)]\(_o\) should stabilize the voltage-sensor proteins of excitation-contraction (E-C) coupling and thereby diminish fatigue (14, 15). Moreover, low [Ca\(^{2+}\)]\(_o\) hastens fatigue induced by repeated tetanic stimulation, as do Ca\(^{2+}\)-channel-blocking agents (32), thus supporting the hypothesis that low [Ca\(^{2+}\)]\(_o\), either directly or in association with other changes does contribute to a reduced force output.

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Most of the work discussed above was performed on isolated amphibian muscle. Some of the conflicting observations mentioned above may be accounted for by differences between amphibian and mammalian muscle. Hence, the main objective of the present study was to examine the role of \([Ca^{2+}]_o\), during fatigue of mammalian skeletal muscle by using contractile experiments. Specifically, we tested the hypothesis that raised \([Ca^{2+}]_o\), contributes to fatigue by examining the following. 1) The effect of altered \([Ca^{2+}]_o\) on the peak force developed by nonfatigued slow- and fast-twitch muscle. 2) The synergistic effect of altered \([Ca^{2+}]_o\) with either elevated extracellular \(K^+\) concentration \([K^+]_o\) and/or lowered extracellular \(Na^+\) concentration \([Na^+]_o\), perturbations thought to contribute to fatigue (14, 15, 23, 29). 3) The effect of altered \([Ca^{2+}]_o\) on fatigue induced by repeated tetanic stimulation. The results show that elevated \([Ca^{2+}]_o\) does not induce fatigue in mammalian muscle but, instead, protects against fatigue; also, they support the hypothesis that repetitive activation may induce fatigue, in part by lowering t-tubular \([Ca^{2+}]_o\). They further show that elevated \([Ca^{2+}]_o\), by hyperpolarizing the sarcolemma, reverses the depolarization blockade of contraction in nonfatigued muscle, induced by elevated \([K^+]_o\), in a manner expected to counteract the effect of elevated \([K^+]_o\) during fatigue.

**MATERIALS AND METHODS**

**Muscle Preparations**

Animal usage and general experimental protocol were approved by the Animal Ethics Committee of Auckland University. Adult female mice (Swiss CD-1), weighing 20–25 g, were killed by cervical dislocation. Intact slow-twitch soleus and fast-twitch extensor digitorum longus (EDL) muscles were dissected in the control Krebs solution (see below), which was gassed with 95% \(O_2\)-5% \(CO_2\) at room temperature. Use of these muscles permitted comparisons to be made with other studies (9, 23, 24).

**Solutions**

The composition of the control Krebs solution, which contained 1.3 mM \(Ca^{2+}\), was (in mM): 122.2 NaCl, 25.1 NaHCO3, 2.8 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.3 CaCl2, and 5 D-glucose; pH was set at 7.4. The measured osmolality was 290 mosmol/l. Solutions with raised \(Ca^{2+}\) concentration \(([Ca^{2+}]_o)\) (5, 10, 15 mM) were made by addition of CaCl2 (with no correction for increased osmolality) to maintain constant \(Na^+\) concentration \([Na^+]_o\). Nominally \(Ca^{2+}\)-free solutions were made by replacing all the \(Ca^{2+}\) with equimolar Mg2+ to prevent depolarization of the sarcolemma (11). These solutions usually contain some \(Ca^{2+}\) (since no EGTA was used; see Ref. 11), and when we changed from the control Krebs solution \(Ca^{2+}\) is likely to have remained in the interfilber space and t tubules. Force recordings and membrane potential \((E_m)\) measurements were made at 25°C. Several experiments were done in elevated \(K^+\) concentration \([K^+]_o\) solutions, in which KCl replaced NaCl. In solutions with reduced \([Na^+]_o\), either chloride chloride (ACROS) or N-methyl-D-glucamine (Sigma Chemical) was used as a replacement for NaCl, as done elsewhere (6). The \([Na^+]_o\) and \([K^+]_o\) in these solutions were checked by flame photometry. The \([Ca^{2+}]_o\) was checked by using a cresolphthalein complexone-GABA-buffered assay on a Hitachi 911 spectrophotometer.

**Contractile Studies**

The methodology used for the contractile experiments (and for \(E_m\) studies and analysis of data) has been described in detail (9). In brief, muscles were suspended vertically and attached to a force transducer in 100 ml of solution in a chamber and immersed in a temperature-controlled water bath. Isometric contractions were evoked by electric field stimulation applied from parallel-plate platinum electrodes. The standard biphasic stimulation pulses (28 V/cm, 0.1 ms) were supramaximal for the twitch in the control Krebs solution (see Fig. 3). Pulse trains were initiated from a computer, and contractions were displayed on an oscilloscope and chart recorder. Selected contractions were stored digitally for further analysis. Maximum tetanic force was evoked at 125 Hz for 2–2.5 s in soleus and at 200 Hz for 1 s in EDL.

The general protocol involved obtaining the optimal muscle length for the twitch followed by equilibration (30–60 min) in the control Krebs solution until a steady-state tetanic force was achieved. Tetani were evoked at 5-min intervals. Contractions were recorded in the control solution, in a modified Krebs solution (i.e., altered \([Ca^{2+}]_o\), \([K^+]_o\), \([Na^+]_o\)) or during fatigue stimulation, and then in the control solution again, to confirm whether any change was reversible. Experimental manipulations such as changing stimulation characteristics or adding salts were done when the tetanic force was at a steady-state level.

In the present study, fatigue is defined as any reversible decline of peak tetanic force induced by repetitive stimulation. There are many different stimulation protocols for inducing fatigue in isolated muscle (31). We used repeated tetanic stimulation separated by short rest periods: each contraction was evoked at 125 Hz for 500 ms, with one contraction induced every second for 100 s. In soleus, these brief contractions produce close to the maximum force in the nonfatigued state, i.e., 94 ± 1% (n = 13), of that achieved with a 2-s tetanus. Mechanical measurements (resting and evoked force) were made during fatiguing stimulation, and in some experiments tetani (with altered stimulation characteristics) were evoked immediately after fatigue (<5 s after; see Figs. 5, 6).

**\(E_m\) Studies**

The \(E_m\) was recorded in surface fibers with standard techniques using glass microelectrodes filled with 3 M KCl (tip resistances of 30–50 MΩ), via an Axoclamp-2 amplifier, and displayed simultaneously on an oscilloscope and a chart recorder. The \(E_m\) was taken as the maximum drop of potential on penetration of the fiber with the potential on withdrawal (up to 3 mV) subtracted for individual recordings. The potential usually returned to zero (set before impalement) after withdrawing the electrode from the fiber. Muscles were incubated in the control Krebs solution for 30–60 min, then exposed to an elevated \([K^+]_o\) solution (containing, randomly, either 1.3 or 10 mM \([Ca^{2+}]_o\)) for at least 55 min. Impalements were made sequentially across the width of the muscle, first at one end and then, following exposure to altered \([Ca^{2+}]_o\), at the other end of the muscle. We have previously shown there to be no difference in the \(E_m\) of fibers penetrated in this way at each end of the muscle (9). A single-blind protocol was employed so that the experimenter recording the \(E_m\) was unaware of which solution was applied to the muscles.

**Analysis**

The force produced in different solutions was expressed relative to the peak tetanic force in the control Krebs solution (evoked with standard pulses of 28 V/cm, 0.1 ms, at 125 Hz in soleus and 200 Hz in EDL). These control tetani give the...
maximum force production (9). The force decline at elevated [K\textsuperscript{+}], was determined by using the control force obtained by correcting for "force rundown," by interpolating between the peak force at 4 mM [K\textsuperscript{+}], immediately before, and with maximum recovery after, increasing [K\textsuperscript{+}]. The peak force during fatiguing stimulation was normalized to the peak force of the first tetanus produced during stimulation, since rundown was negligible over the short duration of the "fatigue run." Muscles were slightly but significantly more fatigable after repeated fatigue runs. On average, the force at the end of fatiguing stimulation fell to 38% of the initial prior to the first fatigue run and to 23% after the fifth. This occurred despite full recovery of peak tetanic force between runs (at least 50 min were allowed between runs). The effect of altered [Ca\textsuperscript{2+}]\textsubscript{o} on fatigue was expressed relative to the mean of control fatigue runs obtained before and after changing the [Ca\textsuperscript{2+}]\textsubscript{o}. The determination of contractile properties, such as the force-frequency relationship and twitch force-stimulation strength relationship, are described elsewhere (9).

Three criteria were used in contractile studies to ensure that the results analyzed came only from healthy preparations. 1) Experiments with rapid (>0.2% min\textsuperscript{-1}) rundown of tetanic force after incubation at elevated [K\textsuperscript{+}], were rejected. The mean recovery of tetanic force was to >95% of the control. 2) Fatigue runs with poor recovery (tetanic force <80% initial) were not used. Tetanic force was potentiated to 109 ± 1% (n = 11) 5-10 min after fatiguing stimulation and reversed within ~35-40 min. The mean recovery of tetanic force following fatigue was to 98 ± 1% (n = 11) of the control. 3) Muscles in which control tetanic force was increased by >20% when stimulated with 1.0-ms pulses rather than control 0.1-ms pulses were rejected. The mean peak tetanic force of the muscles accepted was 127 ± 3 mN (n = 40) for soleus and 188 ± 33 mN (n = 7) for EDL. The EM\textsubscript{a} data of visibly damaged fibers were rejected. Some of the remaining EM\textsubscript{a} data were defined statistically to be extreme outliers and were eliminated post hoc (see Ref. 9).

**Statistical Analyses**

Data are quoted in the text as means ± SE. For contractile data, n is the number of muscles used. For EM\textsubscript{a} data, the number of penetrations is given as number of fibers and number of muscles. Where appropriate, two-way ANOVAs were performed on the data. The level for statistical significance was set at P < 0.05. Only those effects significant at this level are reported in the text.

It proved instructive (see DISCUSSION) to predict the restorative force corresponding to the measured repolarization in the presence of 10 mM [Ca\textsuperscript{2+}]\textsubscript{o} at elevated [K\textsuperscript{+}]. This involved using the previously determined force-resting EM\textsubscript{a} relationships (see Fig. 7 and Ref. 9). Because the measured EM\textsubscript{a} values (at 10 mM [Ca\textsuperscript{2+}]\textsubscript{o}) did not coincide exactly with EM\textsubscript{a} values previously measured in the presence of 1.3 mM [Ca\textsuperscript{2+}]\textsubscript{o}, force was predicted by linear interpolation between adjacent mean EM\textsubscript{a} and force values. The mean predicted force was then compared with the mean measured force, at elevated [K\textsuperscript{+}]\textsubscript{o} with 10 mM [Ca\textsuperscript{2+}]\textsubscript{o}, when using an unpaired t-test (for P < 0.05). The SE of the predicted force was given by the weighted average of the SE values of the adjacent mean measured values.

**RESULTS**

Influence of Changing [Ca\textsuperscript{2+}]\textsubscript{o} on Contraction in Nonfatigued Muscle

We first tested whether raised [Ca\textsuperscript{2+}]\textsubscript{o} contributes to fatigue. This was done by investigating whether elevating [Ca\textsuperscript{2+}]\textsubscript{o} from 1.3 to 10 mM would reduce tetanic force in nonfatigued mammalian skeletal muscle, since force is reduced at this concentration in frog muscle (20, 21). In slow-twitch soleus, exposure to 10 mM [Ca\textsuperscript{2+}]\textsubscript{o}, for 5 min caused a very small but significant decline of peak tetanic force (125 Hz) to 96.2 ± 0.5% (n = 15, range 91.0–98.8%) of the control (see 4K + 10Ca, Fig. 2A). Thereafter, force recovered over a 30- to 40-min period to a steady state and undiminished value of 99.8 ± 0.4%. During such maximal contractions, tropinin-C is thought to be saturated with Ca\textsuperscript{2+} (9), so that any diminution of Ca\textsuperscript{2+} release from the sarcoplasmic reticulum may not affect force. Hence, we investigated submaximal contractions in which negative inotropic effects might be revealed. The steady-state twitch force at 10 mM [Ca\textsuperscript{2+}]\textsubscript{o} was unaffected (99 ± 1%, n = 9, of the control) as were submaximal tetani elicited at 10, 20, and 50 Hz (n = 4). Another possibility is that depressive effects of raised [Ca\textsuperscript{2+}]\textsubscript{o} are confined to fast-twitch mammalian muscle. However, in four fast-twitch EDL, the peak tetanic force (200 Hz) was unchanged: at 5 min it was 99.7 ± 1.2%, and the steady-state value was 102.7 ± 1.9% of the control. We also tested the converse hypothesis that lowered [Ca\textsuperscript{2+}]\textsubscript{o} contributes to fatigue by examining the effect of removing [Ca\textsuperscript{2+}]\textsubscript{o} on contracture of nonfatigued muscle. Soleus muscles were exposed to a nominally Ca\textsuperscript{2+}-free Krebs solution for 20 min, resulting in a small fall of tetanic force to 96.1 ± 0.4% (n = 5) of the control.

Thus neither an increase of [Ca\textsuperscript{2+}]\textsubscript{o} of up to 10 mM nor a very low [Ca\textsuperscript{2+}]\textsubscript{o}, induced large negative inotropic effects in normal solutions in nonfatigued muscle. These results give no indication that altered [Ca\textsuperscript{2+}]\textsubscript{o} per se contributes to fatigue.

Influence of Elevated [Ca\textsuperscript{2+}]\textsubscript{o} on K\textsuperscript{+}-Depressed Contraction

A number of reports suggest that fatigue may result from changes in the distribution of other major ions across the sarcolemma, especially K\textsuperscript{+} (6–9, 14, 29, 31). If this were the case, then raised [Ca\textsuperscript{2+}]\textsubscript{o}, may cause fatigue by acting synergistically with elevated [K\textsuperscript{+}]. Indeed, this idea is attractive, since elevated [Ca\textsuperscript{2+}]\textsubscript{o} inhibits the Na\textsuperscript{+}–K\textsuperscript{+} pump (30) and since inhibition of the Na\textsuperscript{+}–K\textsuperscript{+} pump with ouabain makes the depressive effect of raised [K\textsuperscript{+}] more pronounced (8). Such synergistic effects have already been demonstrated between lowered [Na\textsuperscript{+}]\textsubscript{o} and elevated [K\textsuperscript{+}] (6).

A rise in [K\textsuperscript{+}]\textsubscript{o} from 4 to 10 mM induced a large decline of force in nonfatigued soleus muscle (Fig. 1A, Table 1; see Refs. 7–9). The peak tetanic force evoked at 125 Hz with standard 0.1-ms pulses was reduced to 20% of the control, but the peak force doubled on lowering the frequency to 50 Hz. Furthermore, tetanic force was almost fully restored by using longer (1.0-ms) stimulation pulses (Table 1), which suggests that most of the K\textsuperscript{+}-induced force depression was due to action potential failure. We subsequently exposed the muscles to 10 mM [K\textsuperscript{+}]\textsubscript{o} with 10 mM [Ca\textsuperscript{2+}]\textsubscript{o}, (10K + 10Ca), which, in contrast to the prediction, dramatically restored tetanic force (125 Hz) to ~90% of control (Table 1). This restoration occurred gradually, with maximum...
effects taking ~60 min (see also Fig. 2A). Similarly, the twitch force recovered (Fig. 3A). Potentiating effects were also seen with 10 mM \(\text{Ca}^{2+}\) in \(\text{K}^{+}\)-depressed EDL muscle (Fig. 1B, Table 1). A striking feature with both soleus and EDL was that tetani evoked with 1.0-ms pulses showed little further recovery at elevated \(\text{Ca}^{2+}\). Taken together, these data suggest that in vivo raised \(\text{Ca}^{2+}\) would not act synergistically with a reduced \(\text{K}^{+}\) gradient to cause greater fatigue. On the contrary, it could, conceivably, increase fatigue resistance.

A series of experiments to investigate possible mechanisms for this \(\text{Ca}^{2+}\)-induced restoration of force was performed. The effect of raised \(\text{Ca}^{2+}\), was tested when contraction was abolished at a higher elevated \(\text{K}^{+}\). This force suppression is thought to be due to a failure to generate action potentials (9). Any tetanic force restoration with raised \(\text{Ca}^{2+}\) would then reflect a recovery of excitability rather than \(\text{Ca}^{2+}\) influx through voltage-activated \(\text{Ca}^{2+}\) channels to potentiate E-C coupling. Figure 2 shows that when contraction was completely suppressed at 12 mM \(\text{K}^{+}\) in soleus, there was a huge restoration of tetanic force with 10 mM \(\text{Ca}^{2+}\) to over 70% of the control. The time-dependent responses show that the steady-state force was similar, whether \(\text{Ca}^{2+}\) was added either when force was fully

Table 1. Influence of 10 mM extracellular \(\text{Ca}^{2+}\) concentration on peak force of \(\text{K}^{+}\)-depressed contractions

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Contraction</th>
<th>Solution</th>
<th>4K</th>
<th>10K</th>
<th>10K + 10Ca</th>
<th>[Predicted]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus (n = 5)</td>
<td>Twitch (0.1 ms), %</td>
<td></td>
<td>100</td>
<td>54 ± 11</td>
<td>98 ± 4*</td>
<td>[108 ± 3]</td>
</tr>
<tr>
<td></td>
<td>Tetanus (125 Hz, 0.1 ms), %</td>
<td></td>
<td>100</td>
<td>20 ± 4</td>
<td>89 ± 1*</td>
<td>[85 ± 1]*</td>
</tr>
<tr>
<td></td>
<td>Tetanus (50 Hz, 0.1 ms), %</td>
<td></td>
<td>89 ± 1</td>
<td>39 ± 6‡</td>
<td>85 ± 1*</td>
<td>[85 ± 1]</td>
</tr>
<tr>
<td></td>
<td>Tetanus (125 Hz, 1.0 ms), %</td>
<td></td>
<td>103 ± 2</td>
<td>88 ± 1‡</td>
<td>91 ± 1*</td>
<td>[97 ± 1]</td>
</tr>
<tr>
<td>EDL (n = 4)</td>
<td>Twitch (0.1 ms), %</td>
<td></td>
<td>100</td>
<td>82 ± 8</td>
<td>92 ± 5†</td>
<td>[116 ± 2]†</td>
</tr>
<tr>
<td></td>
<td>Tetanus (200 Hz, 0.1 ms), %</td>
<td></td>
<td>100</td>
<td>44 ± 8</td>
<td>86 ± 3*</td>
<td>[87 ± 2]</td>
</tr>
<tr>
<td></td>
<td>Tetanus (200 Hz, 1.0 ms), %</td>
<td></td>
<td>102 ± 1</td>
<td>55 ± 5‡</td>
<td>63 ± 5</td>
<td>[88 ± 3]</td>
</tr>
</tbody>
</table>

Values are means ± SE for paired data from n muscles. Peak forces are normalized to peak forces in control Krebs solution (4K). 10K and 11K as in the legend of Fig. 1. Stimulation strength: 28 V/cm, frequency and pulse duration as indicated. Tetanus duration: 2–2.5 s for soleus, 1 s for extensor digitorum longus (EDL). [Predicted]: these values in italics are predicted values at 10K + 10Ca determined using force-resting membrane potential relationships (see MATERIALS AND METHODS and DISCUSSION). *Significantly greater (P < 0.05) at elevated extracellular \(\text{K}^{+}\) concentration (4K + 10Ca) to 10 mM \(\text{K}^{+}\) (P < 0.05). †P = 0.141 [note: when using all data, i.e., unpaired as well as paired, peak twitch force for EDL was 80 ± 6% (n = 7) at 11K and 95 ± 4% (n = 6) at 11K + 10Ca; P = 0.052]. ‡Significantly greater than for tetanus at elevated \(\text{K}^{+}\) evoked at 125 Hz for 0.1 ms (soleus) or 200 Hz for 0.1 ms (EDL). $Predicted value significantly different from measured value.
depressed at 12 mM [K\textsuperscript{+}]\textsubscript{o} or simultaneously with 12 mM [K\textsuperscript{+}]\textsubscript{o} (Fig. 2 A). Hence, the reversal and slowing of the K\textsuperscript{+}-induced decline of force are likely to involve the same mechanism. Twitch force also recovered from complete suppression to 95 \pm 6 \% (n = 4). There was no contracture (increase of resting force) on exposure to 10 mM [Ca\textsuperscript{2+}]\textsubscript{o} (with 12 mM [K\textsuperscript{+}]\textsubscript{o}) to indicate a direct effect on E-C coupling independently of action potentials. Moreover, an increase in the stimulation pulse duration from 0.1 to 0.4 or 1.0 ms caused a graded recovery of force at 12 mM [K\textsuperscript{+}]\textsubscript{o} (Fig. 2B), consistent

Fig. 2. Influence of raised [Ca\textsuperscript{2+}]\textsubscript{o} on tetanic contractions at 4 and 12 mM [K\textsuperscript{+}]\textsubscript{o} in soleus. Each point is mean \pm SE (occasionally smaller than symbol). Peak tetanic force was normalized to that for tetanus immediately before changing [Ca\textsuperscript{2+}]\textsubscript{o} or [K\textsuperscript{+}]\textsubscript{o}. A: time course of peak tetanic force at 4K (○), at 4K with 10 mM [Ca\textsuperscript{2+}]\textsubscript{o} (4K + 10Ca; ●, 15 muscles), during exposure to 12 mM [K\textsuperscript{+}]\textsubscript{o} (12K; □), at 12K with 10 mM [Ca\textsuperscript{2+}]\textsubscript{o} (12K + 10Ca; ■), and on return to 4K. Tetani evoked at 125 Hz for 2 s every 5 min. Stimulation pulses: 28 V/cm, 0.1 ms. Steady-state forces obtained either when Ca\textsuperscript{2+} was added simultaneously with 12K at the start (3 muscles) or when added after force was abolished at 12K (4 muscles) were not different (ANOVA). B: influence of raising [Ca\textsuperscript{2+}]\textsubscript{o} from 1.3 to 5–15 mM on tetanic force at 12K. Each data point is from 3–8 muscles. Pulse strength: 28 V/cm, duration and frequency are indicated. Tetanic force (0.1- or 0.4-ms pulses) was significantly greater at 12K with 5–15 mM [Ca\textsuperscript{2+}]\textsubscript{o} compared with that at 12K with 1.3 mM [Ca\textsuperscript{2+}]\textsubscript{o} (ANOVA). Tetanic force (125 Hz, 0.1 ms) was not significantly greater at 10 or 15 mM [Ca\textsuperscript{2+}]\textsubscript{o} (ANOVA). Tetanic force (125 Hz, 1.0 ms) did not significantly increase on raising [Ca\textsuperscript{2+}]\textsubscript{o} (ANOVA).

Fig. 3. Influence of increasing [Ca\textsuperscript{2+}]\textsubscript{o} from 1.3 to 10 mM on twitch force-stimulation strength relationship at 4 and 10 mM [K\textsuperscript{+}]\textsubscript{o} in soleus. Data obtained under steady-state conditions. Each point is mean value with error bars omitted for clarity. Pulse duration: 0.1 ms. Relative peak twitch force at each stimulus strength was expressed as a percentage of that evoked with standard pulses (28 V/cm) at 4K (A). Peak twitch force was expressed as a percentage of that evoked with standard pulses in each solution (B and C). Note, same data are used in A and B. A and B: steady-state relationship at 4K (○; averaged data from before and after change in [K\textsuperscript{+}]\textsubscript{o} and [Ca\textsuperscript{2+}]\textsubscript{o}), at 10K (□), and at 10K + 10Ca (■) from 5 muscles. C: steady-state relationship at 4K and after addition of 10 mM [Ca\textsuperscript{2+}]\textsubscript{o} (4K + 10Ca; ●) from 3 muscles. Note: stimulation strength required to evoke 50% of peak twitch force at 4K + 10Ca (6.1 \pm 0.8 V/cm) was not significantly different to that at 10K + 10Ca (7.4 \pm 0.9 V/cm). Mean twitch force values evoked with standard pulses at 10K and 10K + 10Ca are given in Table 1.
with the force suppression being due to action potential failure. There was no further recovery of tetanic force with 10 mM [Ca\(^{2+}\)], when 1.0-ms pulses were used, and the tetanic force with 0.4-ms pulses increased to a similar level as that achieved with 1.0-ms pulses (Fig. 2B). These results suggest that restorative effects of either raised [Ca\(^{2+}\)], or longer pulses occur via the same processes, i.e., via an effect on action potentials. Moreover, the concentration-response data (Fig. 2B) reveal that a lower [Ca\(^{2+}\)], of 5 mM also increased tetanic force (0.1-ms pulses), with a greater recovery at 50 than at 125 Hz, and that 10 mM [Ca\(^{2+}\)], induced maximal effects.

To check whether the force restoration on addition of CaCl\(_2\) was due to Ca\(^{2+}\), or to Cl\(^-\), we used NaCl to achieve a comparable extracellular Cl\(^-\) concentration as that achieved with CaCl\(_2\). In two soleus muscles incubated at 12 mM [K\(^+\)], the addition of NaCl for up to 25 min failed to restore force (0.1-ms pulses). This result confirmed that the effect of CaCl\(_2\) was not caused or modified by the extra Cl\(^-\) but was due to the raised [Ca\(^{2+}\)].

Force depression at elevated [K\(^+\)] is thought to be due to depolarization of the sarcolemma (7–9, 14). It is possible that the protective effect of raised [Ca\(^{2+}\)], at elevated [K\(^+\)], results from screening of the surface charge, a property shared by divalent cations (12, 25), so that the sarcolemma appears less depolarized (an effect not detectable with microelectrodes; see Ref. 12). Hence we tested whether the divalent cation, Ba\(^{2+}\), could also restore force. Three experiments were done in soleus muscles incubated at either 10 or 12 mM [K\(^+\)], to which BaCl\(_2\) was added to increase the divalent cation concentration to 10 mM. In each case, there was no restoration of force which, thus, eliminated a screening of surface charge as the cause of the force recovery.

Another possibility is that raised [Ca\(^{2+}\)], restores force by reversing the K\(^+\)-induced depolarization (8, 9), since raised [Ca\(^{2+}\)] induces an hyperpolarization in normal solutions (27). Measurement of the E\(_{m}\) demonstrated a significant repolarization with 10 mM [Ca\(^{2+}\)], at elevated [K\(^+\)]. In soleus, the E\(_{m}\) at 10 mM [K\(^+\)], increased by 5.1 mV from −57.1 ± 0.3 mV (56/7 fibers/muscle) to −62.2 ± 0.4 mV (54/7 fibers/muscle) with the additional Ca\(^{2+}\). A similar response occurred in EDL at 11 mM [K\(^+\)], with the E\(_{m}\) increasing by 5.1 mV from −57.7 ± 0.3 mV (49/6 fibers/muscle) to −62.8 ± 0.3 mV (59/6 fibers/muscle) with raised [Ca\(^{2+}\)].

If this 5-mV Ca\(^{2+}\)-induced repolarization causes the restoration of force at elevated [K\(^+\)], then all features of the restored contractions should be similar to those induced with slightly less (5 mV) K\(^+\)-induced depolarization. This proposal was examined specifically by using the twitch force-stimulation strength relationship (Fig. 3). In soleus, exposure to 10 mM [K\(^+\)], caused a right shift of the relationship toward higher stimulation strengths, an effect previously inferred to indicate an increased action potential threshold (9). The E\(_{m}\) at 10K + 10Ca of −62.2 mV can also be induced with a [K\(^+\)], of between 7 and 8 mM at normal [Ca\(^{2+}\)], (9). At a [K\(^+\)], of 8 mM, the twitch force-stimulation strength relationship was unaffected (9), which leads to the prediction that the relationship should also be unaffected at 10K + 10Ca. Figure 3, A and B, shows that, although there was full recovery of the relationship at the higher stimulation strengths (i.e., >12 V/cm), the relationships diverged at lower stimulation strengths. This divergence is apparent at the stimulation strength required to evoke 50% of peak twitch force, which increased from 4.1 ± 0.5 V/cm at 4 mM [K\(^+\)], to 6.5 ± 1.1 V/cm at 10 mM [K\(^+\)], and then increased further to 7.4 ± 0.9 V/cm at 10K + 10Ca. Qualitatively similar shifts were seen in EDL with raised [Ca\(^{2+}\)], at 11 mM [K\(^+\)], (data not shown). The addition of 10 mM [Ca\(^{2+}\)], to the 4 mM [K\(^+\)], control solution induced a similar small right shift of the relationship at the lower stimulation strengths (Fig. 3C): this relationship was not significantly different from that with 10K + 10Ca. Therefore, we attribute the recovery of the relationship at 10K + 10Ca at all except the lower stimulation strengths to the beneficial influence of E\(_{m}\) repolarization on which is superimposed a detrimental effect of raised [Ca\(^{2+}\)], to increase action potential threshold.

**Influence of Elevated \([Ca^{2+}]_o\) on Low-Na\(^+\) Depressed Contraction**

A similar hypothesis to that of the preceding section is that raised [Ca\(^{2+}\)], may contribute to fatigue by acting synergistically with a reduced transsarcolemmal Na\(^+\) gradient. A diminished Na\(^+\) gradient has been shown to occur during fatigue (23, 29), and lowered [Na\(^+\)], also reduces tetanic force in nonfatigued muscle (6, 7). The present experiments examined the effects of reduced [Na\(^+\)], and then raised [Ca\(^{2+}\)], in the presence of reduced [Na\(^+\)], on tetanic force in four nonfatigued soleus muscles (Fig. 4). Lowering of [Na\(^+\)], from 147 to 40 mM (40Na) resulted in a gradual decline of tetanic force to ~40% of the control. The subsequent addition of
10 mM \([\text{Ca}^{2+}]_o\) (40Na + 10Ca) caused a large and immediate (by 5 min) reduction of force to 5% of the control, followed by a small recovery (over 20-30 min) to just over 10% of the control. These data suggest that raised \([\text{Ca}^{2+}]_o\) could contribute to fatigue in vivo when a reduced Na\(^+\) gradient also occurs.

**Effect on Contraction of Changing \([\text{Ca}^{2+}]_o\) With Raised \([\text{K}^+]_o\) and Lowered \([\text{Na}^+]_o\)**

The results of the two preceding sections suggest that raised \([\text{Ca}^{2+}]_o\) may diminish fatigue if the transsarcolemmal K\(^+\) gradient is reduced while contributing to fatigue if the transsarcolemmal Na\(^+\) gradient is reduced. Because these effects are likely to counteract each other to some extent, we investigated the influence of simultaneously changing \([\text{Ca}^{2+}]_o\) with elevated \([\text{K}^+]_o\) and lowered \([\text{Na}^+]_o\) on contraction in nonfatigued soleus muscle. The transsarcolemmal K\(^+\) and Na\(^+\) gradients were changed by extents known to occur during fatigue (14, 23, 29). The \([\text{K}^+]_o\) was doubled to 8 mM and the \([\text{Na}^+]_o\) was reduced by one-third to 100 mM (produces a reduced Na\(^+\) gradient equivalent to a 50% increase of intracellular \([\text{Na}^+]_o\)).

In five muscles exposed to 8 mM \([\text{K}^+]_o\) and 100 mM \([\text{Na}^+]_o\), the tetanic force evoked at 125 Hz fell to 63 ± 6% of the control. The subsequent increase of \([\text{Ca}^{2+}]_o\) to 10 mM induced a significant recovery of force to 82 ± 2% of the control (in ~60 min). Hence, raised \([\text{Ca}^{2+}]_o\) is protective in this situation. Moreover, a reduction in \([\text{Ca}^{2+}]_o\) from 1.8 mM to very low levels (nominally Ca\(^{2+}\) free) in an 8-mM \([\text{K}^+]_o\) and 100-mM \([\text{Na}^+]_o\) solution induced a large decrement of tetanic force (125 Hz) from 71 ± 3 to 23 ± 5% of the control (n = 5) in 20 min. At the low \([\text{Ca}^{2+}]_o\) force was increased by lowering the frequency to 50 Hz, i.e., to 32 ± 6% (n = 5). In two muscles, force was increased to 78 ± 5% of the control when stimulation was achieved with longer pulses (1.0 ms, 125 Hz), which indicates that much of the force depression in this low-[Ca\(^{2+}\)], raised-[K\(^+\)], low-[Na\(^+\)] solution is due to action potential failure. From these data, it would appear that low \([\text{Ca}^{2+}]_o\) is likely to contribute to fatigue by acting synergistically with raised \([\text{K}^+]_o\) and lowered \([\text{Na}^+]_o\).

**Influence of Changing \([\text{Ca}^{2+}]_o\) on Contraction During Intermittent Tetanic Fatigue**

We examined directly the effects of raised \([\text{Ca}^{2+}]_o\) on isometric force during fatiguing stimulation. The “fatigue model” we employed involved the use of whole muscles, a high stimulation frequency, and short rest periods between contractions, i.e., conditions expected to induce large transsarcolemmal ionic changes (24). Force records obtained by using this fatigue protocol with control solutions in soleus demonstrate that fatigue was severe (Figs. 5, A and C, and 6A); the mean decline of tetanic force was to ~40% of the initial value (range 22–50%) (see Fig. 6C, Table 2).

Soleus muscles were exposed to 10 mM \([\text{Ca}^{2+}]_o\) until a steady-state force was achieved (30–40 min) before fatigue was induced. This time was necessary so that central fibers in the muscle were fully exposed to the raised \([\text{Ca}^{2+}]_o\). The subsequent fatigue was notably slower at 10 mM than at 1.3 mM \([\text{Ca}^{2+}]_o\), (Figs. 5 and 6C, Table 2). This attenuation of the decline of peak tetanic force (evoked plus resting) during fatiguing stimulation was ~20% of the initial force or, in other words, amounted to a 30 ± 3% (n = 9) reduction in the fatigue over the 100-s stimulation period. The effect was maximal after ~50 s of stimulation and was maintained until the end of the fatigue run (Figs. 5 and 6C). There appear to be at least two mechanisms responsible for the slowing of fatigue: an increased resting force and an increased evoked force, with each effect contributing about one-half to the total effect (Fig. 5, Table 2). The increase of the resting force at
Mechanism as for the Ca^{2+}-induced restoration of K^-depressed force. When studying contractions evoked a short time (<5 s) after fatiguing stimulation in the control solution, we noticed that a greater force was produced when the frequency was reduced to 50 Hz compared with 125 Hz (Fig. 5, Table 2). In two muscles incubated at 10 mM [Ca^{2+}]_o, it was observed that this difference was absent after fatigue (Fig. 5, Table 2). Furthermore, a most impressive response occurred when we stimulated fatigued muscle with the longer 1.0-ms pulses (125 Hz, 2 s): the decline of tetanic force was almost completely reversed (Fig. 6, Table 2). Table 2 shows that this effect is attributable to the longer stimulation pulses (1.0 vs. 0.1 ms) rather than to the longer stimulation period (2 vs. 0.5 s). Exposure to 10 mM [Ca^{2+}]_o did not modify this response (Table 2).

When soleus muscles were exposed to the nominally Ca^{2+}-free solution for 25 min, they fatigued more quickly (Fig. 6, Table 2). The maximum exacerbation of the fatigue at low [Ca^{2+}]_o was ~14% of the initial tetanic force and occurred toward the end of the stimulation period. This effect amounted to a 23 ± 5% greater extent of fatigue and involved both an attenuation of the increase in the resting force (Fig. 6, Table 2) and a reduction of the evoked force (Fig. 6, Table 2). There was also close to full recovery of tetanic force when stimulation was done with 1.0-ms pulses after fatigue at low [Ca^{2+}]_o (Table 2). In summary, an increase in [Ca^{2+}]_o from a very low level to 10 mM increased tetanic force at the end of fatiguing stimulation from 28 to 55% of the initial force or, in other words, reduced fatigue by ~40%.

### Table 2. Influence of Altered [Ca^{2+}]_o on Contractile Parameters during Intermittent Tetanic Fatigue in Soleus Muscle

<table>
<thead>
<tr>
<th>Fatigue Parameter</th>
<th>[Ca^{2+}]_o, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak tetanic force (at 100 s), %</td>
<td>38 ± 2 (11)</td>
</tr>
<tr>
<td></td>
<td>55 ± 3 (9)*</td>
</tr>
<tr>
<td></td>
<td>28 ± 4 (4)*</td>
</tr>
<tr>
<td>Evoked force (at 100 s), %</td>
<td>32 ± 2 (11)</td>
</tr>
<tr>
<td></td>
<td>41 ± 2 (9)*</td>
</tr>
<tr>
<td></td>
<td>25 ± 4 (4)*</td>
</tr>
<tr>
<td>Resting force (max. increase), %</td>
<td>7 ± 1 (11)†</td>
</tr>
<tr>
<td></td>
<td>17 ± 3 (9)*</td>
</tr>
<tr>
<td></td>
<td>5 ± 1 (4)*</td>
</tr>
<tr>
<td>Resting force (onset time), s</td>
<td>26 ± 1 (11)</td>
</tr>
<tr>
<td></td>
<td>21 ± 2 (9)*</td>
</tr>
<tr>
<td></td>
<td>30 ± 3 (4)*</td>
</tr>
<tr>
<td>125 Hz, 0.5 s, 0.1 ms, %</td>
<td>34 ± 3 (6)</td>
</tr>
<tr>
<td></td>
<td>53 ± 3 (2)</td>
</tr>
<tr>
<td>50 Hz, 0.5 s, 0.1 ms, %</td>
<td>56 ± 4 (6)*</td>
</tr>
<tr>
<td></td>
<td>56 ± 1 (2)</td>
</tr>
<tr>
<td>125 Hz, 2 s, 0.1 ms, %</td>
<td>36 ± 4 (6)</td>
</tr>
<tr>
<td>125 Hz, 2 s, 1.0 ms, %</td>
<td>99 ± 2 (5)*</td>
</tr>
<tr>
<td></td>
<td>94 ± 4 (3)*</td>
</tr>
<tr>
<td></td>
<td>96 ± 3 (2)*</td>
</tr>
<tr>
<td>125 Hz, 2 s, 1.0 ms, %</td>
<td>94 ± 2 (5)</td>
</tr>
<tr>
<td></td>
<td>89 ± 3 (3)</td>
</tr>
<tr>
<td></td>
<td>92 ± 3 (2)</td>
</tr>
</tbody>
</table>

Data are means ± SE. Number of muscles in parentheses. In each case, peak force was expressed relative to the peak force of the 1st tetanus in the train (125 Hz, 0.5 s). Note, peak tetanic force = evoked force + resting force. Stimulation pulses: 28 V/cm, 0.1 ms. Data for tetanus frequency, tetanus duration, and pulse duration shown were obtained within 5 s after fatigue run. *Significantly different (P < 0.05) with altered [Ca^{2+}]_o, from control value at 1.3 mM. †Significant effect during a control fatigue run (P < 0.05). ‡Significantly greater than that evoked at 125 Hz, 0.5 s with 0.1-ms pulses (P < 0.05). §Same data as in preceding line, normalized to control maximum tetanic force evoked at 125 Hz, with 1.0-ms pulses for 2 s in nonfatigued muscle.
DISCUSSION

Do Changes of \([\text{Ca}^2+]_o\) Contribute to Skeletal Muscle Fatigue?

The primary aim of this paper was to resolve the question of whether changes of \([\text{Ca}^2+]_o\) have a role in fatigue of mammalian skeletal muscle. The limited amount of experimental evidence concerning the movement of \([\text{Ca}^2+]_o\) across the sarcolemma during fatigue is conflicting. Some studies suggest that \([\text{Ca}^2+]_o\) increases (3-5, 19, 22) with fatigue, whereas other studies suggest that it decreases (1, 22, 26). The only direct measurements of t-tubular \([\text{Ca}^2+]_o\) during fatigue are in frog muscle, where no change was observed (18).

Possible role of raised \([\text{Ca}^2+]_o\). Our data refute the hypothesis that elevated \([\text{Ca}^2+]_o\) (of up to 10 mM) causes fatigue in mammalian muscle for the following reasons. First, raised \([\text{Ca}^2+]_o\), did not induce a major negative inotropic effect in normal solutions in either nonfatigued slow- or fast-twitch muscle. The small (4%) and transient decline of tetanic force in soleus (Fig. 2A) contrasts with the larger (10-20%) reduction in frog muscle (21). Second, when \([\text{Ca}^2+]_o\) was raised simultaneously with elevated \([\text{K}^+]_o\), and reduced \([\text{Na}^+]_o\) (comparable to the decline of \([\text{K}^+]_o\) and \([\text{Na}^+]_o\) gradients in some types of fatigue; see Refs. 23, 29), force was not further reduced but partially restored. Third, raised \([\text{Ca}^2+]_o\), did not exacerbate intermittent tetanic fatigue (Fig. 5). Fourth, low \([\text{Ca}^2+]_o\), did not attenuate or delay fatigue (Fig. 6). If elevated t-tubular \([\text{Ca}^2+]_o\) contributes to fatigue, it is predicted that low \([\text{Ca}^2+]_o\), would delay stimulation-induced increases of \([\text{Ca}^2+]_o\), and, hence, slow the development of fatigue, but this was not so. Our results suggest, instead, that an elevation of \([\text{Ca}^2+]_o\) provides some protection against fatigue.

Possible role of lowered \([\text{Ca}^2+]_o\). Our results indicate that low \([\text{Ca}^2+]_o\), may contribute to fatigue, but this would require a substantial depletion of \([\text{Ca}^2+]_o\) in the t-tubules. In nonfatigued muscle, nominally \([\text{Ca}^2+]_o\)-free solutions caused only a minor decline of tetanic force (also see Refs. 11, 32), which suggests that low \([\text{Ca}^2+]_o\), per se would not induce much fatigue. We also demonstrated a large synergistic depressive effect between low \([\text{Ca}^2+]_o\), elevated \([\text{K}^+]_o\), and lowered \([\text{Na}^+]_o\). Hence, if the t-tubular \([\text{Ca}^2+]_o\), falls markedly, in association with these other ionic changes, it is likely to contribute to fatigue. Furthermore, intermittent tetanic fatigue was exacerbated with low \([\text{Ca}^2+]_o\), as reported for amphibian muscle (32), and was markedly attenuated with raised \([\text{Ca}^2+]_o\). The more severe fatigue at low \([\text{Ca}^2+]_o\), suggests that the decline of t-tubular \([\text{Ca}^2+]_o\), was even more extreme than at normal \([\text{Ca}^2+]_o\); the slowed fatigue with raised \([\text{Ca}^2+]_o\), may be explained by a better maintenance of t-tubular \([\text{Ca}^2+]_o\), during fatigue.

Our data suggest that an elevation of \([\text{Ca}^2+]_o\), may protect muscle against fatigue and support the hypothesis that a marked reduction in t-tubular \([\text{Ca}^2+]_o\), would contribute to fatigue. However, a direct quantification of t-tubular \([\text{Ca}^2+]_o\), is required during different types and severities of fatigue in mammalian muscle to confirm these suggestions. Until this has been done, any role for \([\text{Ca}^2+]_o\), in fatigue is suggestive. It is possible that \([\text{Ca}^2+]_o\), changes little during fatigue (18) and, therefore, that the modulation of fatigue by experimentally varying \([\text{Ca}^2+]_o\), would reflect some influence on fatigue process(es).

Possible role of \([\text{Ca}^2+]_o\)-independent mechanisms in our model of fatigue. Increase in the \([\text{Ca}^2+]_o\), from 0 to 10 mM reduced the fatigue in our model by ~40% (Fig. 6C); therefore, other mechanisms must be responsible for the remaining fatigue. Several possible \([\text{Ca}^2+]_o\)-independent mechanisms, including reduced transsarcolemmal K⁺ and/or Na⁺ gradients (6-9, 23, 29, 31) and metabolic changes (14, 29, 31), may cause the remaining fatigue.

To assess the importance of changes to the transsarcolemmal K⁺ gradient in our model of fatigue, it is instructive to compare the force generated by nonfatigued muscle at elevated \([\text{K}^+]_o\), with that during fatiguing stimulation (compare Tables 1 and 2). First, fatiguing stimulation reduced tetanic force (125 Hz) considerably (to 40% of initial), as did exposure to 10 mM \([\text{K}^+]_o\), in nonfatigued muscle (to 20% of the control). Second, tetanic force increased by ~20% of the control, on reducing the frequency from 125 to 50 Hz, in both fatigued and K⁺-depressed muscle. Third, during tetanic stimulation in which longer (1.0-ms) pulses were used, force was almost completely restored in both fatigued and K⁺-depressed muscle. The similarity of these effects strongly implies that a reduced K⁺ gradient is a major factor responsible for fatigue in our model. Furthermore, the effect of 10 mM \([\text{Ca}^2+]_o\), on tetanic force at the end of fatiguing stimulation (increase from 35 to 55%, Fig. 6C) is smaller than during exposure to 10 mM \([\text{K}^+]_o\), (increase from 20 to 89%) (see Tables 1 and 2). Hence, we propose that in our model of fatigue any increase of \([\text{K}^+]_o\), is likely to be to <10 mM and that a reduced Na⁺ gradient is possibly also involved.

The mechanism of the impairment in our model of intermittent tetanic fatigue appears to primarily involve the action potential, since almost maximum tetanic force (94%) is generated in fatigued muscle when stimulation is done with the longer 1.0-ms pulses (Fig. 6, Table 2). Regardless of whether the use of 1.0-ms pulses allows action potentials to be restored or whether the pulses directly activate E-C coupling (9), this result implies that virtually all the fatigue can be explained by action potential failure and that any depressive effect of metabolites on cross-bridge function (31) must be small. Our result, however, does not distinguish between action potential failure in the surface or t-tubular membranes. Consistent with our observation is the appearance of inexcitable fibers during a similar fatigue protocol (24). In contrast, some studies on intermittent tetanic fatigue suggest that t-tubular action potentials are unimpaired and that failure is localized to the E-C coupling process at a step beyond charge movement (13, 17, 31). This discrepancy...
may be accounted for by the shorter rest period between contractions in our fatigue model (one tetanus every second), in contrast to the alternative fatigue models, where tetani are evoked every 4–5 s (13, 17, 31). Larger ionic changes are expected with the shorter time for restoration of transsarcolemmal ionic gradients in our fatigue protocol.

Influence of Altered [Ca\(^{2+}\)]\(_o\) on Contraction: Mechanism of Action

Influence of [Ca\(^{2+}\)]\(_o\), on nonfatigued muscle in normal solutions. The lack of any major force decrement with raised [Ca\(^{2+}\)]\(_o\) in the present study, in contrast to frog muscle (20, 21), was presumably because our standard stimulation pulses were markedly supramaximal (see Fig. 3). This is supported by the observation that stimulation with weaker pulses (about one-fourth of our standard stimulation strength) did not have a large effect on twitch force in the control solution but induced a greater (~15%) decline of force at 10 mM [Ca\(^{2+}\)]\(_o\) (n = 2). One effect of raised [Ca\(^{2+}\)]\(_o\) in the control solution was a right shift of the twitch force-stimulation strength relationship toward higher stimulation strengths but only over the lower range of stimulation strengths (Fig. 3C). This response is consistent with the well-known effect (10) of raised [Ca\(^{2+}\)]\(_o\) to elevate the activation threshold for sodium conductance.

Influence of [Ca\(^{2+}\)]\(_o\) in lowered [Na\(^{+}\)]\(_o\) solutions. The mechanism for the extra force decline induced with raised [Ca\(^{2+}\)]\(_o\) in low-Na\(^{+}\)-depressed muscle (Fig. 4) is likely to involve a reduced magnitude of voltage-activated Na\(^{+}\) currents (10) and subsequent impairment of action potential generation and propagation.

Influence of [Ca\(^{2+}\)]\(_o\) in elevated [K\(^{+}\)] solutions. A main finding of the present study is the large restoration of force induced by raised [Ca\(^{2+}\)]\(_o\) in K\(^{+}\)-depressed both slow- and fast-twitch muscles (Figs. 1, 2), which is also seen in rat soleus (T. Clausen, personal communication). This effect could be due to a facilitation of E-C coupling leading to increased intracellular [Ca\(^{2+}\)] (16). However, stimulation with longer (1.0-ms) pulses largely restored force in K\(^{+}\)-depressed muscle, indicative of K\(^{+}\)-induced action potential failure (Table 1, Fig. 2B; see Ref. 9). Subsequent elevation of [Ca\(^{2+}\)]\(_o\) in the solution bathing K\(^{+}\)-depressed muscle selectively restored force when standard 0.1-ms pulses were used but had a negligible further effect with 1.0-ms pulses. In addition, raised [Ca\(^{2+}\)]\(_o\) restored force in muscles made inexorable at 12 mM [K\(^{+}\)]\(_o\), induced a repolarization, and allowed a restoration of action potential threshold (Fig. 3). These combined findings lead us to suggest that an effect on E-C coupling is not involved, but, rather, that raised [Ca\(^{2+}\)]\(_o\) exerts its beneficial effect via the resting potential and ultimately the action potential.

Possible effects of raised [Ca\(^{2+}\)]\(_o\) to increase excitability include: 1) reduced Na\(^{+}\) channel inactivation (27); 2) generation of Ca\(^{2+}\)-dependent action potentials (2); 3) Na\(^{+}\) efflux on the Na\(^{+}\)-Ca\(^{2+}\) exchanger (29) to increase the Na\(^{+}\) gradient (8); or 4) repolarization (present study). We cannot exclude the first three possibilities, but according to the following argument consider that the 5-mV repolarization can account for all of the recovery of force. Using an unpaired t-test (see Methods), we compared the measured force in the presence of 10 mM [Ca\(^{2+}\)]\(_o\) in K\(^{+}\)-depressed muscle with the predicted force values estimated by using the E\(_m\) values measured in the presence of 10 mM [Ca\(^{2+}\)]\(_o\) at elevated [K\(^{+}\)]\(_o\) and our previously established (9) force-resting E\(_m\) relationships (Fig. 7, Table 1). For soleus at 10 mM [K\(^{+}\)]\(_o\), exposure to 10 mM [Ca\(^{2+}\)]\(_o\) caused a repolarization from −57.1 to −62.2 mV, and the measured tetanic force (125 Hz, 0.1-ms pulses) increased from 20 to 89%. This measured force value for −62.2 mV slightly exceeded the predicted value of 85% (see Fig. 7, Table 1). Similarly, the measured restoration of force (0.1-ms pulses) for twitch or tetani evoked at 50 Hz in soleus, or tetani in EDL, was not significantly different from predicted values. However, the tetanic force with 1.0-ms pulses was less than predicted. It is possible that activation of E-C coupling by 1.0-ms pulses, which involves some direct effect (9), rather than activation via the action potential (0.1-ms pulses), is more susceptible to depression by raised [Ca\(^{2+}\)]\(_o\) (25). We conclude that the Ca\(^{2+}\)-induced repolarization reverses the depolarization-induced blockade of action potentials to restore force. The Ca\(^{2+}\)-induced repolarization may be due to a slow entry of Ca\(^{2+}\) into fibers, leading to an increased Ca\(^{2+}\)-activated K\(^{-}\)-conductance (29).

Influence of [Ca\(^{2+}\)]\(_o\) in elevated-[K\(^{+}\)] and lowered-[Na\(^{+}\)] solutions. The force recovery we observed with raised [Ca\(^{2+}\)]\(_o\), at 8 mM [K\(^{+}\)]\(_o\), 100 mM [Na\(^{+}\)]\(_o\) presumably occurred because the protective influence of raised [Ca\(^{2+}\)]\(_o\) at elevated [K\(^{+}\)]\(_o\), dominated over the depressive effect at lowered [Na\(^{+}\)]\(_o\). The effect of low [Ca\(^{2+}\)]\(_o\), at 8 mM [K\(^{+}\)]\(_o\), 100 mM [Na\(^{+}\)]\(_o\) may have arisen after removal of a protective influence of Ca\(^{2+}\) on the resting potential, even at the control [Ca\(^{2+}\)]\(_o\) of 1.3 mM, result-
ing in a further small depolarization that markedly depresses force (Fig. 7).

Influence of \([Ca^{2+}]_o\) on fatigued muscle. A simple interpretation of our fatigue data with altered \([Ca^{2+}]_o\) is that fatigue is partially due to a stimulation-induced lowering of \([Ca^{2+}]_o\) in the t tubules. Modeling work predicts that during fatigue the depletion of \(Ca^{2+}\) would be greater deep within the t tubules (1), as with Na\(^+\) depletion and K\(^+\) accumulation (7, 13, 31). This leads to the suggestion that fatigue processes are localized to t-tubular membranes. Indeed, low \([Ca^{2+}]_o\) may act synergistically to reduce force with an activity-dependent increase of \([K^+]_o\) and/or reduction of \([Na^+]_o\), which can occur in the t tubules (14, 29, 31). Alternatively, fatiguing stimulation may make some cellular process susceptible to low \([Ca^{2+}]_o\).

Raised \([Ca^{2+}]_o\) slowed fatigue by increasing both the resting and evoked tetanic forces (Fig. 5). This increase of resting force was linked to incomplete relaxation between tetani and is likely to be a consequence of elevated resting intracellular \([Ca^{2+}]_o\), perhaps due to enhanced \(Ca^{2+}\) influx via the Na\(^+\)-\(Ca^{2+}\) exchanger (29) or through voltage-activated \(Ca^{2+}\) channels (2, 16, 28). The increased evoked force with raised \([Ca^{2+}]_o\) during fatigue may be due to mechanism(s) similar to those that restored force in \(K^+\)-depolarized muscle, i.e., by attenuating depolarization and maintaining excitability. In addition, raised \([Ca^{2+}]_o\) may increase \(Ca^{2+}\) influx during fatigue to maintain E-C coupling and tetanic intracellular \([Ca^{2+}]_o\) when the normal \(Ca^{2+}\) release is impaired during fatigue (16). Conversely, the detrimental effects of low \([Ca^{2+}]_o\) on fatigue may involve attenuation of these \(Ca^{2+}\)-induced protective processes.

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