Effects of modulators of sarcoplasmic Ca\(^{2+}\) release on the development of skeletal muscle fatigue

E. Germinario,\(^1\) A. Esposito,\(^1\) A. Megighian,\(^1\) M. Midrio,\(^1\) R. Betto,\(^2\) and D. Danieli-Betto\(^1\)

\(^1\)Department of Human Anatomy and Physiology, University of Padova; and \(^2\)Consiglio Nazionale delle Ricerche Neuroscience Institute, Muscle Biology and Physiopathology Unit, 35121 Padova, Italy

Submitted 7 May 2003; accepted in final form 13 October 2003

MUSCLE FATIGUE IS DEFINED as the failure to maintain the expected or required power output (9). Many factors operate together to cause this failure, the relative importance of each being dependent on the intensity, duration, and nature of the exercise; on the composition of the motor unit involved; and on muscle training.

A reduction of myoplasmic Ca\(^{2+}\) level due to an impairment of sarcoplasmic reticulum (SR) Ca\(^{2+}\) release is considered one of the major causes of muscle fatigue (1, 5, 11, 32, 33). Indeed, a progressive reduction in myoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)), accompanying the loss of muscle tension, has been observed under a variety of experimental fatigue conditions (2, 5, 31). The reduced release of Ca\(^{2+}\) from the SR is largely consequent to the profound alterations of metabolite levels caused by the intense muscle work. The accumulation and/or exhaustion of various metabolites not only influence SR Ca\(^{2+}\) release and uptake, but also the sensitivity to Ca\(^{2+}\) of myofibrillar proteins and the tension-generating capacity of contractile elements (1, 32).

Dantrolene is a muscle relaxant, known to decrease contractile force (12) by affecting Ca\(^{2+}\) release from the SR (13, 21, 23–25). The resulting reduction of twitch force is produced without affecting contractile proteins, sliding filament interaction, SR Ca\(^{2+}\) pump activity (7), or membrane properties (10, 30). Many similarities have been reported between the effects of dantrolene and those of fatigue on contractile properties of skeletal muscle (16, 20), reinforcing the view that reduction of SR Ca\(^{2+}\) release participates in causing muscle fatigue. On the other hand, caffeine is a drug known to facilitate Ca\(^{2+}\) release from the SR, thereby increasing [Ca\(^{2+}\)], and force production (3).

If changes in [Ca\(^{2+}\)]\(_{i}\) play a role in the development of muscle fatigue, the presence of drugs known to affect [Ca\(^{2+}\)], should be expected to modify the time course and the degree of tension failure. In the present work, we examined whether the presence of drugs that modulate Ca\(^{2+}\) release from the SR, such as dantrolene and caffeine, could counteract or enhance the development of fatigue. Indeed, both drugs were able to modify fatigue development. However, on the whole, the results indicate that the changes induced by caffeine and dantrolene on fatigue profile are mainly due to the changes in the initial level of tetanic tension caused by the drugs, rather than to drug-dependent changes in the SR Ca\(^{2+}\) release during fatigue development.

MATERIALS AND METHODS

All the experiments were carried out in accordance with the Helsinki Accords for Humane Treatment of Animals during Experimentation. The experimental plan was approved by the Ethics Committee of the Medical Faculty of the University of Padova. This study was performed by using muscles isolated from Swiss mice 3–4 mo of age.

Experimental procedures. The animals were killed with a high dose of ether. Soleus and extensor digitorum longus (EDL) muscles were quickly isolated and immersed in a solution of the following composition (in mM): 119.9 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 3.2 MgCl\(_2\), 1.3 NaH\(_2\)PO\(_4\), 2.5 NaHCO\(_3\), 11.1 glucose; pH 7.2–7.4. The solution was continuously bubbled with 95% O\(_2\)-5% CO\(_2\). Muscles were then mounted vertically in a bath filled with the same solution, with added 30 µM d-tubocurarine, at 30°C. Muscles were electrically stimulated with supramaximal pulses (0.5-ms duration) delivered by a Grass S44 electronic stimulator through a stimulus-isolation unit (Grass SIU5), and isometric force was recorded with a Grass FT03 force transducer.
Two-dimensional analysis of MLCs. PLC composition was analyzed by two-dimensional gel electrophoresis as previously described (6). Briefly, 20 cryostat muscle sections (20 μm) were taken from control and 20 μM dantrolene-treated EDL muscles. The sections were dissolved in 100 μl of 9.5 M urea, 2% (vol/vol) Nonidet NP-40, 5% (vol/vol) 2-mercaptoethanol, 1.0% (vol/vol) Ampholine (American Pharmacia) of pH range 5–7, and 1.0% (vol/vol) Ampholine of pH range 3.5–10, and subjected to isoelectric focusing. The second dimension of the gel consisted of SDS-PAGE in 15% (wt/vol) polyacrylamide slab gels. The gels were then stained with silver staining, and the relative amount of each protein band (MLC isoforms) was determined by densitometry using a Bio-Rad imaging densitometer (GS-670).

Statistical analysis. Values are presented as means ± SE. The statistical significance was tested with Student’s t-test for unpaired samples. Differences were considered significant at the P < 0.05 level.

RESULTS

Effects of dantrolene and caffeine on twitch tension. Dantrolene affected contractile properties of both soleus and EDL muscles. In the presence of 5 or 20 μM dantrolene, twitch tension was reduced by 32.4 ± 1.4 or 68.6 ± 2.7%, respectively, in EDL muscle, and by 20.3 ± 6.8 or 47.6 ± 4.3% in soleus muscle. The presence of 2 mM (in soleus) and 5 mM caffeine (in EDL) significantly increased the amplitude of the twitch (21.1 ± 4.6% in EDL, n = 4, and 14.0 ± 2.5% in soleus muscles, n = 4). Caffeine (either 2 or 5 mM) did not induce contracture in resting muscles.

Effects of dantrolene on EDL muscle fatigue. In control EDL muscle, twitch tension developed during the fatiguing protocol progressively diminished so that, at the end of stimulation, the fatigue index was 0.388 ± 0.029 (Fig. 1). In the presence of 5 μM dantrolene, the initial 60-Hz tetanic tension decreased significantly (P < 0.01), from 7.2 ± 0.7 N/g in control (n = 4) muscles to 5.0 ± 0.3 N/g with 5 μM dantrolene (n = 6), whereas the time course of fatigue was not affected (fatigue index = 0.357 ± 0.024; Figs. 1 and 2A). In the presence of 20 μM dantrolene, the initial tension was markedly (P < 0.001) reduced (2.1 ± 0.1 N/g, n = 7) with respect to the control values. However, during the first 3 min of stimulation, tension gradually increased, demonstrating a positive staircase which provided a measure of the percent tension decline resulting from the fatigue protocol.

In selected experiments designed to test whether myosin light chain (MLC)-2 phosphorylation influenced fatigue, the MLC-2 kinase inhibitor ML-7 hydrochloride (Calbiochem) was employed.

Effects of dantrolene on twitch tension. The presence of 2 mM (in soleus) and 5 mM caffeine (in EDL) significantly increased the amplitude of the twitch (21.1 ± 4.6% in EDL, n = 4, and 14.0 ± 2.5% in soleus muscles, n = 4). Caffeine (either 2 or 5 mM) did not induce contracture in resting muscles.

Effects of dantrolene on EDL muscle fatigue. In control EDL muscle, twitch tension developed during the fatiguing protocol progressively diminished so that, at the end of stimulation, the fatigue index was 0.388 ± 0.029 (Fig. 1). In the presence of 5 μM dantrolene, the initial 60-Hz tetanic tension decreased significantly (P < 0.01), from 7.2 ± 0.7 N/g in control (n = 4) muscles to 5.0 ± 0.3 N/g with 5 μM dantrolene (n = 6), whereas the time course of fatigue was not affected (fatigue index = 0.357 ± 0.024; Figs. 1 and 2A). In the presence of 20 μM dantrolene, the initial tension was markedly (P < 0.001) reduced (2.1 ± 0.1 N/g, n = 7) with respect to the control values. However, during the first 3 min of stimulation, tension gradually increased, demonstrating a positive staircase which

Fig. 1. Fatigue development in extensor digitorum longus (EDL) muscle. Recordings were obtained from control vehicle-treated muscles (●, n = 3) and from muscles preincubated with 5 (○, n = 4) and 20 μM dantrolene (□, n = 4), 20 μM dantrolene + 15 μM ML-7 (▲, n = 4), and 5 mM caffeine (○, n = 4). *P < 0.05. **P < 0.005, with respect to the control, §P < 0.05 with respect to 20 μM dantrolene.

Fig. 2. Effects of 5 μM dantrolene (A), 20 μM dantrolene (B), and 5 mM caffeine (C) on contractile responses during fatiguing stimulation in EDL muscle. Gray traces are from the treated muscles.
displayed a 60% increase in tension by the end of third minute. Thereafter, tension gradually decreased, reaching the initial level at the end of stimulation (fatigue index \(0.811 \pm 0.044, P < 0.0001\) with respect to controls; Figs. 1 and 2B). Importantly, 15 \(\mu M\) ML-7 nearly abolished the ability of dantrolene to produce the staircase, and no signs of fatigue were evident (Fig. 1).

**Effects of dantrolene on soleus muscle fatigue.** In soleus muscle, preincubation with either 5 or 20 \(\mu M\) dantrolene did not significantly modify the initial tetanic force \(8.2 \pm 0.8\ N/g\) in control \((n = 3), 9.5 \pm 1.0\ N/g\) in 5 \(\mu M\) dantrolene \((n = 3), \) and \(8.1 \pm 0.6\ N/g\) in 20 \(\mu M\) dantrolene \((n = 5))\]. The progressive decline of tetanic tension during the fatiguing stimulation was unaffected with 5 \(\mu M\) dantrolene but significantly slowed in the first 2–3 min with 20 \(\mu M\) dantrolene (Figs. 3 and 4). Fatigue indexes at the sixth minute time points were not modified in the presence of both 5 \(\mu M\) \((0.58 \pm 0.02)\) and 20 \(\mu M\) dantrolene \((0.58 \pm 0.04)\) with respect to controls \((0.57 \pm 0.02)\).

**Effects of caffeine on EDL and soleus muscles fatigue.** Caffeine caused a significant \((P < 0.05)\) increase of tetanic tensions in both muscles \((10.4 \pm 0.6\ N/g\) in EDL, \(n = 4,\) and \(13.8 \pm 1.6\ N/g\) in soleus muscles, \(n = 4)\). During fatiguing stimulation, tension of both EDL (Figs. 1 and 2C) and soleus (Figs. 3 and 4C) muscles declined with a steeper slope than in controls. Fatigue indexes at the sixth minute time points were \(0.31 \pm 0.02\) in 5 \(mM\) caffeine-treated EDL muscle \((P < 0.05\) with respect to control) and \(0.53 \pm 0.01\) in 2 \(\mu M\) caffeine-treated soleus muscle.

**DISCUSSION**

Intense and prolonged exercise produces profound modifications of intracellular muscle milieu, leading to the accumulation and the exhaustion of metabolites, which eventually cause the progressive decline of tension production \((3, 17, 27)\). Contraction-dependent factors of fatigue cause tension loss by affecting SR \(Ca^{2+}\) release, sensitivity to \(Ca^{2+}\) of myofibrillar proteins, and tension-generating capacity of contractile elements, with the reduced \([Ca^{2+}]_{i}\) being considered a major contributor to muscle fatigue \((4, 11, 27, 32)\). Here, we studied whether the presence of drugs, caffeine and dantrolene, that modulate \(Ca^{2+}\) release from the SR is able to counteract or to enhance the development of fatigue. In effect, both drugs modified the development of fatigue. However, as a whole, our results indicate that the modifications induced by the two drugs are attributable more to the changes in the initial level of tetanic tension than to changes in SR \(Ca^{2+}\) release during fatigue development.

In EDL muscle, dantrolene caused a reduction of twitch and tetanic tensions, both at 5 \(\mu M\) and, more intensely, at 20 \(\mu M\). The 5 \(\mu M\) dantrolene did not affect the timing and size of fatigue, whereas 20 \(\mu M\) dantrolene completely abolished the development of fatigue, and, on the contrary, produced a transient potentiation of tension. The lack of fatigue in the
The presence of 20 μM dantrolene can be reasonably attributable to the strong reduction of the initial tetanic tension, and as a consequence to the smaller accumulation of contraction-dependent factors. It is, however, possible that fatigue development is masked by tension potentiation (17, 26). However, in the presence of ML-7, a specific inhibitor of MLCK that eliminated potentiation, no signs of fatigue were evident. It is worth noting that tension potentiation during intermittent stimulation of fast muscle has been attributed to increased phosphorylation of MLC-2 (14, 28), causing a leftward shift of the pCa-tension curve, i.e., an increased Ca\(^{2+}\) sensitivity of myofibrillar elements (29). The positive effect on tension is better appreciated when [Ca\(^{2+}\)] \(_i\) is low and tension at the beginning of stimulation is lower than that observed when maximally activated by Ca\(^{2+}\) (22). This explains the observation that dantrolene facilitates the occurrence of the phenomenon (22), as also confirmed by our experiments. Our results, showing that tension potentiation during stimulation was accompanied by an increase in the level of phosphorylated MLC-2, are in agreement with other data in the literature (18). Moreover, the observation that ML-7 prevents tension potentiation provides a support to the hypothesis concerning the cause of positive staircase.

From our results with high dantrolene concentration, it appears that, in the absence of an adequate initial contraction tension level, the deficiency of myoplasmic Ca\(^{2+}\) is not sufficient per se to induce the development of fatigue.

The lack of effects by 5 μM dantrolene on the development of fatigue in EDL muscle apparently does not support the importance of the strength of contraction, and of Ca\(^{2+}\) deficiency, in causing fatigue. The lower initial tetanic tension should produce reduced contraction-dependent changes of metabolites and then a lower or slowed development of fatigue with respect to the controls. On the other hand, the reduction of initial muscle strength depends only on the reduced SR Ca\(^{2+}\) release caused by the drug (13, 21, 23–25). It may be that, in the presence of 5 μM dantrolene, the effect of reduced contraction-dependent changes of metabolites was enhanced by the reduced availability of myoplasmic Ca\(^{2+}\), resulting in a tension decline during stimulation greater than that expected on the basis of the initial muscle strength. By this point of view, the reduced [Ca\(^{2+}\)] \(_i\) seems to have a role in facilitating the development of fatigue.

The presence of caffeine produced an initial higher tetanic tension in soleus and EDL muscles. It is thus apparent that during the development of fatigue in the presence of caffeine, the higher [Ca\(^{2+}\)] \(_i\) level is not protective against the negative action of the larger changes of fatigue metabolites.

In soleus muscle, neither 5 nor 20 μM dantrolene reduced the initial tetanic force, although both drug concentrations were effective in reducing twitch tension. The lower ability of dantrolene to affect tetanus tension with respect to twitch tension was also noted by others (15). Thus, in soleus muscle, dantrolene appears less effective in affecting SR Ca\(^{2+}\) release under tetanic stimulation. It is therefore not surprising that fatigue indexes with dantrolene were similar to those of untreated soleus. However, evidence for an action of dantrolene also during tetanic contractions comes from our observation that 20 μM dantrolene significantly slowed fatigue development during the first minutes of stimulation. This slowing effect may indicate that, at the beginning of the fatiguing process, there could be an excess of [Ca\(^{2+}\)] \(_i\), in the muscle, with a negative effect on tension-generation capability, and that dantrolene improved contractile activity by reducing Ca\(^{2+}\) release. It is of interest that the occurrence of a [Ca\(^{2+}\)] \(_i\) increase during the initial phases of a fatiguing stimulation protocol, contrasting with the decrease of developed tension, has been actually demonstrated in both amphibian (2) and mammalian (8, 31) single skeletal muscle fibers.

REFERENCES


