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High-frequency fatigue of skeletal muscle: role of extracellular Ca^{2+}

Elena Germinario,

Department of Human Anatomy and Physiology, University of Padova, Via Marzolo 3, 35131 Padova, Italy

Istituto Interuniversitario di Miologia, Padova, Italy

Alessandra Esposito,

Department of Human Anatomy and Physiology, University of Padova, Via Marzolo 3, 35131 Padova, Italy

Menotti Midrio,

Department of Human Anatomy and Physiology, University of Padova, Via Marzolo 3, 35131 Padova, Italy

Samantha Peron,

Department of Human Anatomy and Physiology, University of Padova, Via Marzolo 3, 35131 Padova, Italy

Philip T. Palade,

Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

Romeo Betto, and

Istituto Interuniversitario di Miologia, Padova, Italy

Neuromuscular Biology and Physiopathology, C.N.R. Neuroscience Institute, Viale G. Colombo 3, 35121 Padova, Italy

Daniela Danieli-Betto

Department of Human Anatomy and Physiology, University of Padova, Via Marzolo 3, 35131 Padova, Italy

Istituto Interuniversitario di Miologia, Padova, Italy

Daniela Danieli-Betto: daniela.danieli@unipd.it

Abstract

The present study evaluated whether Ca^{2+} entry operates during fatigue of skeletal muscle. The involvement of different skeletal muscle membrane calcium channels and of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) has been examined. The decline of force was analysed in vitro in mouse soleus and EDL muscles submitted to 60 and 110 Hz continuous stimulation, respectively. Stimulation with this high-frequency fatigue (HFF) protocol, in Ca^{2+} -free conditions, caused in soleus muscle a dramatic increase of fatigue, while in the presence of high Ca^{2+} fatigue was reduced. In EDL muscle, HFF was not affected by external Ca^{2+} levels either way, suggesting that external Ca^{2+} plays a general protective role only in soleus. Calciseptine, a specific antagonist of the cardiac isoform ($\alpha 1\text{C}$) of the

dihydropyridine receptor, gadolinium, a blocker of both stretch-activated and store-operated Ca^{2+} channels, as well as inhibitors of P2X receptors did not affect the development of HFF. Conversely, the Ca^{2+} ionophore A23187 increased the protective action of extracellular Ca^{2+} . KB-R7943, a selective inhibitor of the reverse mode of NCX, produced an effect similar to that of Ca^{2+} -free solution. These results indicate that a transmembrane Ca^{2+} influx, mainly through NCX, may play a protective role during HFF development in soleus muscle.

Keywords

Dihydropyridine receptors; Stretch-activated Ca^{2+} channels; Store-operated Ca^{2+} channels; P2X receptors; $\text{Na}^+/\text{Ca}^{2+}$ exchanger

Introduction

Muscle contraction is triggered by an increase in concentration of myoplasmic Ca^{2+} released from sarcoplasmic reticulum (SR). In skeletal muscle Ca^{2+} release from the SR is dependent on conformational interaction between the activated voltage-gated Ca^{2+} channel, the dihydropyridine receptor (DHPR), and the SR Ca^{2+} -release channel (Lamb 2000). However, there is some evidence that skeletal muscle contraction can be modulated by extracellular Ca^{2+} . Elevation of extracellular Ca^{2+} level protects against fatigue produced by brief repeated tetani at low frequency (Cairns et al. 1998).

The aim of the present work was to further investigate the role of extracellular Ca^{2+} in fatigue development. The study was carried out in high-frequency fatigue (HFF), which is an experimental form of muscle fatigue that is a model for a sustained maximum voluntary effort (Cairns and Dulhunty 1995). HFF is ascribed to reduction of Ca^{2+} release from the SR, as a consequence of a reduction of action potential amplitude and propagation caused by the anomalous distribution of Na^+ and K^+ ions across the T-tubule membranes (Cairns and Dulhunty 1995; Fitts 1994; Westerblad et al. 1991). The rise of $[\text{K}^+]_o$ and $[\text{Na}^+]_i$ during the high-frequency stimulation indicates that the Na^+-K^+ pump capacity is limited, particularly in the T-tubules where the pump density is lower than that in sarcolemma (Fitts 1994). During recovery, the Na^+-K^+ pump rapidly reversed the anomalous distribution of the two ions.

It is possible that extracellular Ca^{2+} could play a protective role during fatiguing stimulation either by entering the muscle fibre through some membrane channels and/or by acting on undefined extracellular sites. Consistent with the first possibility is the presence of a number of cell membrane Ca^{2+} channels in skeletal muscle both in the plasma membrane and in the T-tubules (even though their physiological role is far from clear) and that sustained skeletal muscle activity stimulates Ca^{2+} uptake into the fibre (Gissel and Clausen 1999, 2000).

Besides serving an understandable role in the refilling of depleted SR, Ca^{2+} entry during muscle contraction activity could exert diverse additional functions, not all positive. Ca^{2+} entry might be associated with the general regulation of gene expression and with the local modulation of signalling pathways (Bassel-Duby and Olson 2006). Ca^{2+} entry might, however, also activate proteases to contribute to fatigue (Verburg et al. 2006) and favours the delayed loss of membrane integrity (Gissel and Clausen 2000, 2003). The use of HFF protocol, characterized by rapid onset of and recovery from fatigue (seconds), should exclude the long-term effects of Ca^{2+} entry and reveal its possible short-time effects.

Ca^{2+} entry from the extracellular medium is not essential to stimulate contraction of skeletal muscle (Lamb 2000), as the skeletal muscle DHPR isoform ($\alpha 1\text{S}$) behaves as a voltage sensor rather than as a Ca^{2+} channel. However, we have shown that discrete amounts of the cardiac

$\alpha 1C$ isoform are also expressed in the T-tubules of slow skeletal muscle (Pereon et al. 1997, 1998). It is thus possible that entry of Ca^{2+} through the $\alpha 1C$ isoform may play some role during contraction as well as sustained activity.

Store-operated, voltage independent, Ca^{2+} channels (SOCs) have been described in skeletal muscle, i.e. channels that are activated upon depletion of intracellular Ca^{2+} stores. The cumulative entry of Ca^{2+} through SOCs not only may represent a mechanism for refilling of the SR, but may also provide additional Ca^{2+} needed for muscle contraction under conditions of intensive exercise and fatigue (Ducret et al. 2006; Ma and Pan 2003). Recently, it has been shown that Ca^{2+} influx through SOCs occurs shortly after initiation of SR depletion (Launikonis and Rios 2007). Stretch-activated cation channels (SACs) are also present in skeletal muscle cell membrane (Franco Jr and Lansman 1990; Ducret et al. 2006). The mechanical stimulation of skeletal muscle membrane during contractile activity thus may provide enough activation of SACs to cause the entry of Ca^{2+} as well as other monovalent and divalent cations (Franco Jr and Lansman 1990). Recent evidence identifies TRPC1, a cation channel member of the TRP family (transient receptor potential), as the protein that seems to form both SOCs and SACs (Vandebrouck et al. 2002), as also suggested by the overlapping sensitivity to pharmacological agents. Even though it is not known how TRPC1 could be sensitive to both store depletion and membrane stretch, it appears related to its localization in the T-tubule membranes. Importantly, inhibition of such channels increased sensitivity to fatigue, further suggesting that Ca^{2+} entry might play a role in maintaining force (Ducret et al. 2006).

Recently, extracellular ATP-operated P2X receptors have been described in skeletal muscle (Ryten et al. 2001; Sandonà et al. 2005). These receptors are cationic channels that when activated by ATP become permeable mainly to Ca^{2+} , but also to Na^+ and K^+ , according to the intra/extracellular gradient of these ions (North 2002). Stimulated skeletal muscle fibres release ATP in the extracellular milieu causing the autocrine/paracrine activation of P2X receptors located at the cell membrane of the fibres (Cunha and Sebastiao 1993; Sandonà et al. 2005). As a consequence, a discrete influx of Ca^{2+} through these channels is also likely to occur during HFF. Importantly, we have recently shown that ATP-mediated Ca^{2+} entry, most likely through a P2X₄ receptor located in the T-tubules, plays an important role in modulating contractility of skeletal muscle during prolonged stimulation (Sandonà et al. 2005).

Na^+/Ca^{2+} exchanger (NCX) is known to play a significant role in maintaining skeletal muscle Ca^{2+} homeostasis (Frayssé et al. 2001). NCX is a protein responsible for transmembrane Ca^{2+} movement directly linked to reciprocal movement of Na^+ . Depending on the electrochemical gradients of Na^+ and Ca^{2+} , NCX provides either Ca^{2+} extrusion (normal mode) or entry (reverse mode) (Blaustein and Lederer, 1999). Three NCX isoforms have been cloned so far, with NCX1 and NCX3 being expressed in skeletal muscle (Frayssé et al. 2001). Importantly, a higher sensitivity to fatigue was demonstrated in the NCX3-knock out mouse (Sokolow et al. 2004).

Our results show that external Ca^{2+} plays a protective role versus HFF of the slow-contracting, fatigue resistant, soleus muscle, whereas in the fast-contracting, fatigue sensitive, EDL muscle it seems less important. The beneficial role of external Ca^{2+} appears to predominantly depend on transmembrane influx through the NCX.

Materials and methods

The study and the protocols utilized were approved by the Ethics Committee of the Medical Faculty of the University of Padova and by the Health Ministry of Italy.

The soleus and EDL muscles (weighing 8.6 ± 0.2 mg, $n = 59$, and 12.5 ± 0.7 mg, $n = 13$, respectively) utilized in the study were taken out from Swiss CD-1 mice (3 months old, weighing 43.2 ± 0.8 g, $n = 40$), sacrificed by cervical dislocation. The experiments were performed in vitro in a vertical muscle apparatus (300B, Aurora Scientific Inc, Canada) containing a Ringer solution of the following composition: 120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 3.15 mM MgCl_2 , 1.3 mM NaH_2PO_4 , 25 mM NaHCO_3 , 11 mM glucose, 30 μM *d*-tubocurarine, pH 7.2–7.4, 30°C, bubbled with 95% O_2 –5% CO_2 . The muscles were stretched to the optimal length (i.e. the length that allowed maximal tension development in response to a single pulse) and electrically stimulated, by two parallel electrodes, with supramaximal pulses (0.5 ms duration) delivered by a Grass S44 electronic stimulator through a stimulus isolation unit (Grass SIU5). Muscle response was recorded through an isometric force transducer (Grass FT03) connected to an AT-MIO 16AD acquisition card (National Instruments) and data were analysed by a specific module of the National Instruments Labview software (Danieli-Betto et al. 2005).

Fatiguing protocol

The muscle was first stimulated to record twitch tension. Then a first fatiguing (HFF) protocol was applied to the muscle. After 10-min rest, a second twitch was evoked to verify the complete recovery of muscle tension after the fatiguing stimulation. Then, the muscle was subjected to the diverse treatments described below, after which twitch tension was measured again. Finally, a second HFF stimulation was applied to the muscle (Fig. 1). In a series of experiments, soleus muscle was pre-incubated with the selected drug before the second HFF in 5 mM Ca^{2+} (effect of treatments in high calcium). The HFF protocol consisted of a prolonged tetanus at stimulation frequency (60 and 110 Hz for soleus and EDL, respectively) that allowed the initial development of about 80–85% of the maximal tension. The characteristics of tetanic tension decline during the HFF protocol were evaluated by the following parameters: P_{hff} , the maximal tension developed during HFF; FT_{25} and FT_{50} , the time necessary to cause 25 and 50% decline of the maximal tension, respectively; FI, the fatigue index, i.e. the ratio between the tension developed at the end of HFF stimulation (after 40 and 8 s for soleus and EDL, respectively) and the maximal tension P_{hff} (Fig. 1). The different stimulation frequencies applied to EDL and soleus muscles are dependent on the specific tetanic fusion frequency and sensitivity to fatigue of the two muscles. However, in some experiments, soleus muscle was stimulated using the same fatiguing protocol as that used for EDL (110 Hz for 20 s). To evaluate the effects of the different treatments applied to the muscles, the HFF parameters before and after every single treatment were compared.

Treatments

Preliminary experiments were performed to determine the appropriate treatment, drug concentration and incubation time. The following treatments were performed: (1) control condition: after the first HFF stimulation the muscle was incubated for 10 min in Ringer solution before evoking the second HFF; (2) nominally Ca^{2+} -free solution: the muscle was incubated for 5 min in a modified Ringer solution in which calcium was substituted with an equivalent concentration of MgCl_2 ; (3) high-calcium solution: CaCl_2 was added to the Ringer solution to the final concentration of 5 mM; the muscle was incubated for 15 min; (4) drug-containing Ringer: (a) the Ca^{2+} ionophore A23187 (20 μM) (Calbiochem, CA, USA) incubated for 15 min; (b) calciseptine (4 μM , Alomone, Israel) incubated for 10 min; (c) gadolinium (Gd^{3+}) (20 μM) incubated for 10 min; (d) mixture of P2X receptors inhibitors composed of 20 μM pyridoxalphosphate-6-azophenyl-2',5'-disulphonic acid (PPADS, Tocris Cookson, Avonmouth, UK), 10 μM suramin (Sigma) and 50 μM reactive blue-2 (Sigma-RBI) incubated for 10 min; (e) KB-R7943 mesylate (20 μM) (Calbiochem, CA, USA) incubated for 15 min. In addition, all the treatments described at point 4) have also been performed in high-calcium Ringer. Each muscle was subjected to only one treatment.

Statistical analysis

Mean and standard error of the mean (SEM) were calculated according to standard procedures. The statistical significance of differences between means was determined by using the Student's paired *t*-test. Statistical significance was set at $P < 0.05$.

Results

We hypothesized that Ca^{2+} entry could be relevant to skeletal muscle fibre fatigability. To test this hypothesis, we evaluated the effects of various treatments on twitch tension and on the development of HFF. As a demonstration of the appropriateness of the recovery time utilized between the two HFF stimulation, twitch tension as well as the HFF parameters were almost identical in the control condition (Table 1, Table 2, Table 3).

Effect of Ca^{2+} -free Ringer

First, we investigated the effects of Ca^{2+} -free Ringer on twitch tension. The low $[\text{Ca}^{2+}]_o$ caused about a 21% reduction ($P < 0.05$) of soleus twitch tension (Table 1), while that of EDL was almost unmodified (Table 2). Similarly, low $[\text{Ca}^{2+}]_o$ produced large effects on HFF development in soleus (Table 1 and Table 3) and none in EDL (Table 2). In fact, the HFF parameters of soleus, P_{hff} , FT_{25} , FT_{50} and FI, were significantly reduced in Ca^{2+} -free Ringer (Table 1 and Table 3; and Fig. 2).

Effect of high extracellular Ca^{2+} levels

Twitch tension and P_{hff} of soleus (Table 1) and of EDL muscles (Table 2) were not affected by high $[\text{Ca}^{2+}]_o$. In contrast, high $[\text{Ca}^{2+}]_o$ alleviated soleus muscle fatigue (Fig. 2), as indicated by the significantly higher FT_{25} and FI values (Table 3), while it was ineffective on the HFF development of EDL muscle (Table 2). To verify whether these effects were produced by the different stimulation frequency (60 Hz in soleus and 110 Hz in EDL), the soleus muscle was also stimulated at 110 Hz. The results confirm the protective action of raised Ca^{2+} , in fact, after 20 s HFF stimulation at 110 Hz, FI was 0.25 ± 0.05 and 0.37 ± 0.03 in 2.5 mM Ca^{2+} and in 5 mM Ca^{2+} , respectively ($n = 4$, $P < 0.02$).

Effects of various treatments in normal Ca^{2+}

Ca^{2+} ionophore A23187—To test the effects of higher Ca^{2+} influx, we utilized A23187, an agent that favours the entry of Ca^{2+} into the muscle cells without causing muscle contraction and leakage of plasma membrane (Gissel and Clausen 2003). Twitch tension and P_{hff} of soleus muscle (Table 1) were not significantly affected by A23187 (Table 1), while it markedly influenced the fatigue development (Fig. 2). In fact, FT_{25} and FI were significantly increased in presence of the Ca^{2+} ionophore A23187 (Table 3).

Calciseptine—Since the absence of extracellular Ca^{2+} produced a significant increase of HFF in soleus muscle, not evident in EDL muscle, we hypothesized that soleus muscle could utilize the DHPR $\alpha 1\text{C}$ isoform to compensate for any possible Ca^{2+} loss or waste. We then tested this possibility by using calciseptine, a specific antagonist of this Ca^{2+} channel in the heart (de Weille et al. 1991). Twitch tension of soleus muscle was unaffected by calciseptine while it caused a diminution of P_{hff} (Table 1). Calciseptine had no effects on the HFF development of soleus muscle (Table 3).

Gadolinium—It is known that gadolinium is a potent inhibitor of mechano-sensitive SACs (Yang and Sachs 1989) and of SOC (Putney et al. 2001), an action also demonstrated in skeletal muscle (Ducret et al. 2006). Gadolinium (20 μM) was ineffective on soleus twitch tension (Table 1), and on HFF parameters (Table 3).

Inhibitors of P2X receptors—Since several isoforms of P2X receptor channels are expressed in skeletal muscle (Ryten et al. 2001; Sandonà et al. 2005), we examined the possible involvement of P2X purinergic receptors as mediators of Ca^{2+} entry during HFF. To assess this possibility, we utilized a mixture of three P2X inhibitors: 20 μM PPADS, 10 μM suramin and 50 μM reactive blue-2 (North 2002; Sandonà et al. 2005). This inhibitor cocktail was ineffective on twitch tension and on P_{hff} (Table 1). On the other hands, these inhibitors slowed HFF development (higher FT_{25}) of soleus muscle (Table 3). Since inhibitors of P2X receptor channels, besides that of Ca^{2+} , may also block Na^{+} and K^{+} fluxes across the membrane, we also tested their action in EDL muscle, where extracellular Ca^{2+} was without evident effects. Interestingly, inhibition of P2X receptors significantly increased FT_{50} and FI (Table 2).

KB-R7943—A total of 20 μM KB-R7943 mesylate, a selective inhibitor of the reverse mode action of the NCX (Naro et al. 2003) produced effects in soleus muscle analogous to those of Ca^{2+} -free condition (Table 1 and Table 3). KB-R7943 mesylate caused 18 and 28% reduction ($P < 0.05$) of P_t and P_{hff} , respectively (Table 1). Moreover, the drug markedly influenced fatigue development. In fact, FT_{25} , FT_{50} and FI were significantly reduced in presence of KB-R7943 (Table 3 and Fig. 2).

Effects of various treatments in high calcium

In order to evaluate whether the beneficial effects of high extracellular Ca^{2+} during HFF are mediated by Ca^{2+} influx, we carried out the same treatments described above in the presence of 5 mM Ca^{2+} . The effects were evaluated on the FI fatigue index. As reported in Table 3, high Ca^{2+} produced a large protective effect on HFF with respect to normal Ca^{2+} (+52.9%, Table 4). The high Ca^{2+} effect was even higher in the presence of Ca^{2+} ionophore A23187 (+76.8%), while it was slightly attenuated in the presence of calciseptine, Gd^{3+} , and inhibitors of P2X receptors (+30.7, +35.6 and +24.8%, respectively). In the presence of NCX inhibitor KB-R7943, the protective effect produced by 5 mM Ca^{2+} was severely attenuated (+7.8%), so that the FI value was only slightly larger than that measured in 2.5 mM Ca^{2+} (Table 4).

Discussion

This work demonstrates that extracellular Ca^{2+} is important for sustaining contractile responses during HFF development, an experimental form of muscle fatigue that has been compared to a sustained maximum voluntary effort (Cairns and Dulhunty 1995). The dependence on external Ca^{2+} is evident in the slow-twitch, fatigue resistant, soleus skeletal muscle, but not in the fast-twitch, fatigue sensitive, EDL muscle. This could indicate that external Ca^{2+} is a causal factor of the lesser fatigability of slow skeletal muscles with respect to fast muscles. Extracellular Ca^{2+} could play a protective role during HFF either by entering the muscle fibre and/or by acting on undefined extracellular sites. Our results show that Ca^{2+} entry, mainly through the NCX, reduces fatigue development.

Our results confirm that low external Ca^{2+} concentrations induced a reduction of twitch and tetanic tension of soleus muscle (Dulhunty and Gage 1988). Since we have demonstrated the presence of the $\alpha 1\text{C}$ isoform of DHPR in adult rat soleus muscle, but not in EDL muscle (Pereon et al. 1998), we hypothesized that Ca^{2+} influx may occur through this Ca^{2+} channel. Our experiments with calciseptine, a drug that specifically blocks the $\alpha 1\text{C}$ isoform of DHPR (de Weille et al. 1991), seem to confirm this hypothesis, since it did inhibit the maximal tension induced during HFF (lower P_{hff} in Table 1). A recent study on skeletal muscle showed that calciseptine did not affect twitch and tetanic tensions; however, this result was obtained in fast-twitch muscles (Garcia et al. 2001) that do not express the $\alpha 1\text{C}$ isoform (Pereon et al. 1998). Our results also show that high concentration of extracellular Ca^{2+} and the Ca^{2+} ionophore A23187 did not affect peak tension, and this lack of effects suggests that the action of

extracellular calcium is already maximal at physiological concentrations and that its action could only be reduced in Ca^{2+} -free conditions.

The absence of extracellular Ca^{2+} also modified the profile of HFF development, largely worsening fatigue of soleus muscle, but not of EDL. The opposite manoeuvre, high extracellular Ca^{2+} concentration, reduced the HFF of soleus muscle. Calciseptine failed to influence HFF development, indicating that Ca^{2+} influx through the $\alpha_1\text{C}$ isoform is not involved in tension decline during HFF. It has been reported that low extracellular Ca^{2+} concentration reduces the intra-membranous charge movements within the DHPR, while high calcium levels stimulate them, suggesting that extracellular Ca^{2+} has a stabilizing action on the DHPR (for references see Fitts 1994). Thus, it is also possible that an increase in T tubular Ca^{2+} reduces fatigue by stabilizing the DHPRs.

We considered that activation of SOC, SAC and/or P2X receptor channels, expressed by skeletal muscle, might contribute to the entry of Ca^{2+} affecting HFF development. However, Gd^{3+} , inhibitor of SOC and SAC, was ineffective on HFF development. This result could be explained by a moderate SR Ca^{2+} depletion during HFF so that the contribution of SOC is almost undetectable. Consistently, maximum K^+ contractures can still be generated during severe HFF, suggesting that high Ca^{2+} levels are still available in the SR (Cairns and Dulhunty 1995). Finally, SACs could be not sufficiently stretched during the isometric contraction of HFF, confirming that they are mainly activated during eccentric contraction (Franco Jr and Lansman 1990).

The possible role of P2X receptors is consistent with the liberation of the P2X agonist, ATP, during the tetanic contractions (Sandonà et al. 2005). Therefore, we expected that treatment with P2X receptors inhibitors, by preventing activation of these channels, would induce an effect similar to that of Ca^{2+} -free solution. On the contrary, inhibitors of P2X receptors reduced HFF. It is worth considering that P2X receptors are non-specific cationic channels (North 2002), i.e. not only permeable to Ca^{2+} , but also to Na^+ and K^+ . Moreover, the main cause of HFF has been identified as a reduced action potential amplitude and propagation as a consequence of the failure of K^+ and Na^+ ions to redistribute across the T-tubule membranes during the repetitive stimulation (Cairns and Dulhunty 1995; Fitts 1994; Williams and Ward 1991; Dutka and Lamb 2007). Thus, the build-up of ions in the limited T-tubules luminal space has the effect of rapidly compromising excitation–contraction coupling and reducing Ca^{2+} release from the SR. The inhibition of P2X receptor cationic channels during HFF blocks the influx of Ca^{2+} but also that of Na^+ as well as the efflux of K^+ , thus reducing cell membrane depolarization. As a consequence, the generation and propagation of potentials during HFF is favoured, and tension is better maintained (Cairns and Dulhunty 1995). It is possible that the physiological influx of Ca^{2+} through these channels during HFF makes tension decline less noticeable, but this positive action of Ca^{2+} is continuously counteracted by the altered distribution of Na^+ and K^+ . The inhibition of P2X receptor cationic channels abolishes Ca^{2+} , Na^+ and K^+ fluxes through these channels, attenuating the protective effects due to the influx of Ca^{2+} , as well as the fatiguing effects caused by the redistribution of Na^+ and K^+ ions across the T-tubule membranes. This hypothesis is partly supported by the results in EDL muscle, where extracellular Ca^{2+} was ineffective, while the blockade of P2X channels reduced HFF.

Our results seem to indicate that in physiological conditions (2.5 mM Ca^{2+}) extracellular Ca^{2+} produces some protective effects during HFF by entering the cell mainly through NCX. In fact by blocking the NCX we have an effect similar to that of Ca^{2+} -free. It is possible that the reverse mode action of NCX plays a physiological role in attenuating tension decline during HFF. This is based on the fact that on cell membrane depolarization, the reverse mode of NCX is activated and contributes to Ca^{2+} entry into the muscle fibre (Blaustein and Lederer 1999).

Besides NCX, we imagine that the contribution of the other membrane Ca^{2+} channels is too small to produce significant effects, detectable with our method in physiological Ca^{2+} . In fact, when higher levels of external Ca^{2+} were available (5 mM) during HFF, the protective effects of Ca^{2+} entry are enhanced in the presence of the Ca^{2+} ionophore but always counteracted, at least in part, by blocking the individual channels (Table 4). Once again, the NCX inhibitor produced the greatest effect. However, this inhibitor did not completely abolish the action of elevated extracellular Ca^{2+} . Additional experiments were performed in which the muscle was first fatigued in 2.5 mM Ca^{2+} and then in 5 mM Ca^{2+} , both tests performed in the presence of KB-R7943. The mean FI value of the first HFF in normal Ca^{2+} (+KB-R7943) was 0.285 ± 0.03 , while the FI value of the second HFF in high Ca^{2+} , in spite of the presence of KB-R7943, was slightly higher [0.336 ± 0.05 ($n = 3$), +18%] with the difference being significant ($P < 0.037$). As with the results in Table 4, this result suggests that KB-R7943 blocks the majority, but not all of the Ca^{2+} influx in high Ca^{2+} . There are two possible interpretations for failure to inhibit all the action of elevated Ca^{2+} , which our data cannot resolve: (1) other Ca^{2+} permeable channels may contribute significantly, particularly at 5 mM Ca^{2+} , or (2) the effects of the drug and 5 mM external Ca^{2+} are antagonistic but possibly in part via different mechanisms, such as some additional strictly extracellular action of Ca^{2+} not blocked by KB-R7943.

The lack of effects due to extracellular Ca^{2+} in EDL could be explained by published data showing that inactivation of the DHPR voltage-sensor occurs with depolarization in skinned fast-twitch fibres (Lamb 2002). Thus, it is likely that inactivation of DHPR is more important during HFF in EDL than in soleus, concealing any effects produced by changed extracellular Ca^{2+} levels.

In conclusion, our results show that in soleus muscle extracellular Ca^{2+} plays a positive effect on the resistance to fatigue, mainly by a transmembrane influx, while its absence reduces it. In fact, the effects demonstrated by the Ca^{2+} ionophore A23187 and the NCX reverse mode inhibitor KB-R7943 in high extracellular Ca^{2+} clearly indicate that Ca^{2+} influx plays a more relevant role than just the simple elevation of extracellular Ca^{2+} .

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Fig. 1.

Typical HFF stimulation protocol. Muscles were set to the optimal length and then the first twitch was evoked (**a**). Successively, one continuous tetanus (110 Hz in EDL muscle shown) was evoked (**b**). After 10 min rest, a time sufficient for the complete muscle recovery, a second twitch was recorded (**c**). The solution was then replaced by a fresh one containing the selected drug (in the control example, no drug was added) and the muscle was incubated for the required time. At the end of incubation, the muscle was stimulated to evoke a further twitch (**d**). Finally, a second fatiguing tetanus was produced (**e**). To measure the effects produced by the selected drug, the following parameters were considered: twitch tension (P_t) measured in **d** was compared to that in **c**; tetanus amplitude measured after incubation (**e**) was compared to that before incubation (**b**); P_{hff} , value of maximal tension, FT_{25} and FT_{50} times for 25 and 50% decline of P_{hff} maximal tension, P_{end} tension at the end of stimulation protocol

Fig. 2.

Representative HFF traces of soleus muscle. Soleus muscle HFF recordings were produced by continuous stimulation at 60 Hz for 40 s. Force is normalized to peak tetanic force (P_{hff}). With reference to the stimulation protocol of Fig. 1, only the second HFF trace (e) is shown. Before the HFF stimulation shown, soleus muscle was incubated under either one of the following conditions: 10 min in normal Ringer solution (control); 5 min in low Ca^{2+} Ringer (Ca^{2+} -free); 5 min in Ringer with 5 mM Ca^{2+} (high Ca^{2+}); 15 min in Ringer with 20 μ M A23187 Ca^{2+} ionophore (A23187); 15 min in Ringer with 20 μ M KB-R7943. The *dashed lines* represent 25% (P_{25}) and 50% (P_{50}) of the maximal tension (P_{hff}) that were utilized to calculate the corresponding T_{25} e T_{50} values

Table 1

Twitch and high-frequency fatigue tensions of soleus muscle

Treatments	P_t (N/g)		P_{hff} (N/g)	
	Before	After	Before	After
Control ($n = 4$)	1.61 ± 0.23	1.56 ± 0.24	12.9 ± 1.1	12.4 ± 1.0
Ca^{2+} -free ($n = 5$)	1.82 ± 0.12	$1.44 \pm 0.20^*$	14.5 ± 1.2	$11.5 \pm 1.2^*$
5 mM Ca^{2+} ($n = 5$)	1.55 ± 0.08	1.45 ± 0.08	13.1 ± 1.0	13.1 ± 0.8
20 μM A23187 ($n = 7$)	1.30 ± 0.13	1.29 ± 0.14	10.6 ± 1.4	10.8 ± 1.2
4 μM calciseptine ($n = 5$)	1.66 ± 0.19	1.57 ± 0.19	14.1 ± 1.3	$13.1 \pm 1.2^*$
20 μM Gd^{3+} ($n = 13$)	1.46 ± 0.11	1.51 ± 0.11	13.7 ± 1.3	12.7 ± 5.0
P2X inhibitors ($n = 10$)	1.49 ± 0.06	1.51 ± 0.88	11.8 ± 1.0	11.7 ± 0.8
20 μM KB-R7943 ($n = 6$)	1.89 ± 0.13	$1.67 \pm 0.15^*$	19.7 ± 0.9	$15.2 \pm 1.2^*$

Values are mean $\pm$ SEM. Each muscle was submitted to only one treatment. In parenthesis is the number of muscles examined

P_t twitch tension developed before and after the indicated treatments (see Sect. "Methods"), P_{hff} maximal tetanic tension developed during the HFF stimulation protocol (see Fig. 1)

* $P < 0.05$, indicates the statistical significance with respect to the values before the relevant treatment

Table 2

Twitch tension and high-frequency fatigue properties of EDL muscle

Treatments	P_t (N/g)		P_{hff} (N/g)		FT_{50} (s)		FI	
	Before	After	Before	After	Before	After	Before	After
Control ($n = 3$)	1.55 ± 0.06	1.47 ± 0.04	10.6 ± 1.3	9.6 ± 1.0	3.9 ± 0.3	4.0 ± 0.2	0.220 ± 0.031	0.240 ± 0.030
Ca ²⁺ -free ($n = 3$)	1.40 ± 0.18	1.38 ± 0.19	10.6 ± 0.9	9.5 ± 1.3	4.4 ± 0.5	4.7 ± 0.2	0.297 ± 0.025	0.299 ± 0.032
5 mM Ca ²⁺ ($n = 3$)	2.04 ± 0.05	1.94 ± 0.03	12.4 ± 1.1	11.7 ± 1.0	4.0 ± 0.3	4.0 ± 0.6	0.233 ± 0.032	0.240 ± 0.033
P2X inhibitors ($n = 4$)	1.45 ± 0.31	1.44 ± 0.28	11.3 ± 1.7	10.4 ± 1.4	3.4 ± 0.5	3.8 ± 0.4*	0.239 ± 0.054	0.289 ± 0.054*

Values are mean ± SEM. In parenthesis is the number of muscles examined

P_t twitch tension developed before and after the indicated treatments, P_{hff} maximal tetanic tension developed during the HFF stimulation protocol (see Fig. 1), FT_{50} time for the 50% decline of maximal tension, and FI the ratio between tension developed at the end (P_{end} in Fig. 1) of HFF stimulation (after 8 s) and P_{hff}

* $P < 0.05$ indicates the statistical significance with respect to the values before the relevant treatment

Table 3

High-frequency fatigue properties of soleus muscle

Treatments	FT ₂₅ (s)		FT ₅₀ (s)		FI	
	Before	After	Before	After	Before	After
Control (n = 4)	12.6 ± 1.8	14.2 ± 3.1	24.1 ± 3.6	25.6 ± 4.7	0.279 ± 0.067	0.287 ± 0.077
Ca ²⁺ -free (n = 5)	15.1 ± 2.5	9.1 ± 1.1 *	27.8 ± 2.8	19.2 ± 1.4 *	0.334 ± 0.055	0.192 ± 0.041 *
5 mM Ca ²⁺ (n = 5)	14.1 ± 0.7	25.9 ± 4.1 *	27.6 ± 1.2	n.a.	0.325 ± 0.033	0.497 ± 0.077 *
20 μM A23187 (n = 7)	17.3 ± 1.7	24.4 ± 2.1 *	28.0 ± 2.0	n.a.	0.359 ± 0.037	0.439 ± 0.049 *
4 μM calciseptine (n = 5)	15.5 ± 1.6	17.1 ± 4.1	25.3 ± 1.2	27.2 ± 1.3	0.305 ± 0.006	0.306 ± 0.002
20 μM Gd ³⁺ (n = 13)	20.4 ± 6.1	18.5 ± 2.0	n.a.	n.a.	0.355 ± 0.035	0.337 ± 0.035
P2X inhibitors (n = 10)	12.8 ± 0.9	16.6 ± 1.6 *	23.6 ± 1.6	n.a.	0.261 ± 0.030	0.309 ± 0.046
20 μM KB-R7943 (n = 6)	23.2 ± 3.4	17.3 ± 3.2 *	29.4 ± 1.9	22.6 ± 1.5 *	0.348 ± 0.041	0.245 ± 0.030 *

Values are mean ± SEM. In parenthesis is the number of muscles examined

FT₂₅ and FT₅₀ time for the 25 and 50% decline of maximal tension (in some experiments FT₅₀ was not reached before the end of the analysis, 40 s, so that the mean was not calculated and it was indicated as n.a., not applicable), FI the ratio between tension developed at the end (P_{end} in Fig. 1) of HFF stimulation (after 40 s) and P_{Hff}

* P < 0.05 with respect to the values before the relevant treatment

Table 4

Effects of treatments on the protective action produced by high extracellular Ca^{2+} (5 mM) during high-frequency fatigue of soleus muscle

Treatment	FI		
	Before in 2.5 mM Ca^{2+} only	After in 5 mM Ca^{2+} + treatment	Δ (%)
None ($n = 5$)	0.325 ± 0.033	$0.497 \pm 0.077^*$	+52.9
20 μM A23187 ($n = 4$)	0.298 ± 0.042	$0.527 \pm 0.070^*$	+76.8
4 μM calciseptine ($n = 4$)	0.293 ± 0.055	$0.383 \pm 0.032^*$	+30.7
20 μM Gd^{3+} ($n = 5$)	0.331 ± 0.019	$0.449 \pm 0.052^*$	+35.6
P2X inhibitors ($n = 10$)	0.382 ± 0.039	$0.477 \pm 0.042^*$	+24.8
20 μM KB-R7943 ($n = 7$)	0.346 ± 0.022	0.373 ± 0.031	+7.8

Values are mean $\pm$ SEM

The fatigue index (FI) indicates the ratio between tension developed at the end of HFF stimulation (after 40 s, P_{end} in Fig. 1) and the maximal tension P_{hff} developed during stimulation. The FI value generated during the first HFF stimulation in normal Ca^{2+} (2.5 mM) only is compared to the value obtained in the HFF stimulation in high Ca^{2+} (5 mM) either with or without the indicated treatments. The percent increment [Δ (%)] between the two HFF stimulations is also given. In parenthesis is the number of muscles examined

* $P < 0.05$