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ORIGINAL RESEARCH

Mitochondrial K_{ATP} channels in skeletal muscle: are protein kinases C and G, and nitric oxide synthase involved in the fatigue process?

This article was published in the following Dove Press journal:
Open Access Animal Physiology
24 October 2012
Number of times this article has been viewed

Elizabeth Sánchez-Duarte[1]
Xóchitl Trujillo[1]
Miguel Huerta[1]
Mónica Ortiz-Mesina[1]
Christian Cortés-Rojo[2]
Salvador Manzo-Ávalos[2]
Alfredo Saavedra-Molina[2]
Rocío Montoya-Pérez[3]

[1]Unidad de Investigación Enrico Stefani Bonfanti, Centro Universitario de Investigaciones Biomédicas, Universidad de Colima, Colima, México; [2]Instituto de Investigaciones Químico-Biológicas, [3]Coordinación General de Estudios de Posgrado Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México

Background: Fatigue in skeletal muscle is defined as a reduction in the physical power needed to execute a function or as an inability to maintain mitochondrial ATP production. The mitochondrial potassium channel (mitoK_{ATP}) participates in combating fatigue in skeletal muscle. In this work, we evaluated the role of the mitoK_{ATP} channel activator (diazoxide) and inhibitors of the signaling routes (protein kinase C, staurosporine; protein kinase G, KT5823; and nitric oxide synthase, metil N^G-Nitro-L-arginine ester, L-NAME), on muscle fatigue tension. In addition, we evaluated the main signaling routes used by the nitric oxide synthase protein and protein kinase C and G, in the presence of their specific activators.

Methods: We used the anterior latissimus dorsi skeletal muscle of 2–3-week-old chicks. This muscle consists of slow muscle fibers. Tension was achieved by applying repetitive electrical stimulation that induced fatigue in an in vitro model.

Results: Diazoxide significantly reduced muscle fatigue ($P = 0.0002$ in peak tension, $P = 0.000002$ in maximum tension) by increasing post-fatigue tension, in spite of the fact that 5-hydroxydecanoate, a selective inhibitor of mitoK_{ATP}, did not suppress post-fatigue tension.

Conclusion: Our results suggest a lack of direct interaction in inhibition of the signaling routes during fatigue-induced mitoK_{ATP} activation. This effect is possibly due to the type of skeletal muscle fibers (slow), the stimulation protocols (twitch), and the animal (avian) model used in the study.

Keywords: fatigue, skeletal muscle, mitochondrial ATP-sensitive potassium channels

Introduction

ATP-dependent potassium channels (K_{ATP}) were described in patches or membrane derived from guinea pig ventricular myocytes.[1] These channels have been reported in several tissues, including β-pancreatic cells,[2] skeletal muscle fibers of amphibians,[3] mammals,[4–6] and birds,[7,8] and in the liver-derived internal membrane mitochondria of the rat.[9] K_{ATP} channels connect cell excitability with its metabolism and play an important role in several cellular functions by detecting the intracellular ATP/ADP relationship.[10]

Mitochondrial K_{ATP} (mitoK_{ATP}) has been isolated and partially purified, and its function in mitochondrial swelling has been demonstrated.[9,11] It is composed of a sulfonylurea receptor, coupled with an inward-rectifying potassium channel (Kir 6.x), and configured in a stoichiometry of 4:4.[12] The skeletal muscle K_{ATP} channel phenotype varies in its molecular composition, biophysical properties, and pharmacological response depending on the type of muscle.[5,6] Garlid et al[13] demonstrated that mitoK_{ATP} activation by diazoxide, a specific activator, was greater than sarcolemmal K_{ATP} activation.

Correspondence: Rocío Montoya-Pérez
Coordinación General de Estudios de Posgrado, Universidad Michoacana de San Nicolás de Hidalgo, Francisco J Mújica s/n
Ciudad Universitaria, Edif C5,
Col Felícitas del Río 58030,
Morelia, Michoacán, México
Tel +52 44 3322 3500 ext 4157
Email biochio@yahoo.com.mx

Some of the activators or inhibitors of the mitoK$_{ATP}$ channel affect the activity of protein kinase C,[14] such as 5-hydroxydecanoate, chelerythrine, or calphostine C, which inhibit the beneficial effect of diazoxide on pretreated mitochondria during preconditioning to ischemia.[15] Protein kinase C modifies excitation-contraction coupling by phosphorylation of Ca^{2+} L-type channels and/or ryanodine receptors,[16] whereas activation of mitoK$_{ATP}$ activates the downstream protein kinase C pathway, leading to production of reactive oxygen species.[16] These signalizing routes could be involved in the activity of mitoK$_{ATP}$ in relation to muscle fatigue, because there is a functional level of interaction between mitoK$_{ATP}$ and protein kinase C.[17] Direct phosphorylation of the channel results in a conformational change that opens the channel and fluxes K^+ to the mitochondria. Costa et al[18] and Costa and Garlid[19] reported on protein kinase G activation and mitoK$_{ATP}$ channel opening, suggesting that there was an increase in production of reactive oxygen species. Light scattering measurements in mitochondria swelling tests indicate that cGMP-dependent protein kinase (protein kinase G) induces a similar opening in mitoK$_{ATP}$, as do K$_{ATP}$ activators (diazoxide and cromakalim) in the heart, liver, and brain-derived mitochondria. Costa et al[18] also reported inhibition of this effect by the K$_{ATP}$ inhibitor (5-hydroxydecanoate), the lipophilic quaternary ion (tetraphenyl phosphonium), sulfonylurea (glibenclamide), and the protein kinase G inhibitor (KT5823). These results suggest that protein kinase G could be the cytosolic component of the mitoK$_{ATP}$ activation route, transmitting the protection signal from the cytosol to the inner mitochondrial membrane through a route that involves one of the kinase proteins (the PKC-ε).[19]

On the other hand, there is evidence indicating direct mitoK$_{ATP}$ activation by nitric oxide,[20,21] and it has been suggested that protein kinase C and nitric oxide synthase are intermediaries in the signalizing route that activates mitoK$_{ATP}$ in fatigue-induced mammalian skeletal muscle.[22] Therefore, we conducted a study using an in vitro model to determine the participation of mitoK$_{ATP}$ in fatigue of avian slow skeletal muscle fibers using activators and inhibitors of this channel and evaluated the proposed signalization routes related to this channel.

Materials and methods

Ethical approval

Chickens were used in accordance with the Institute for Laboratory Animal Research[23] Guide for the Care and Use of Laboratory Animals and the Ethics Committee of the Centro Universitario de Investigaciones Biomedicas of the Universidad de Colima, which approved the protocol. To minimize animal pain and distress and to perform muscle extraction, the chicks were anesthetized beforehand with chloroform, followed by cervical dislocation and decapitation, ensuring a fast and complete separation of the head from the body, according to the American Veterinary Medical Association guidelines on euthanasia.[24]

Dissection

The anterior latissimus dorsi muscle was used for this investigation because it is exclusively composed of slow muscle fibers. The muscles were carefully dissected out, together with a piece of the humerus bone and a portion of the proximal tendon, from 2–3-week-old chicks. The spinal cord was pinned to the bottom of the experimental chamber and 3.0 surgical silk threads were tied around the humerus bone to attach the distal end of the muscle bundle (1–2 mm thick) to a force transducer (Grass FT03, West Warwick, RI) by means of a lightweight wire. To record the force, a mechanical transducer was wired to an amplifier (Cyberamp 320; Axon Instruments, Foster City, CA), and connected to an analog-to-digital converter (Digidata 1322A; Axon Instruments) with a 5 Hz sampling rate. Data were acquired using the Axoscope subroutine (pClamp 9.2; Axon Instruments) on a desktop computer.

Fatigue protocols

We used different electrical stimulation frequencies to produce twitches to establish a fatigue protocol in the slow latissimus dorsi muscle, as previously described.[25] In short, supramaximal twitches at 300 ms and 0.2 Hz (Grass S-48 stimulator and SIU-5B stimuli isolation unit) were applied until bundle contractions reduced the control contraction by approximately 50% (at about 60 minutes). Pulses were delivered across platinum wire electrodes situated on both sides of the muscle. Each protocol consisted of an initial stimulation period to induce fatigue (about 50% of initial tension), after which drugs were applied to the bath for 6 minutes to maintaining stimulation. Drug withdrawal was then carried out by passing normal Ginsborg saline[26] through the muscle and continuing electrical stimulation for 15 minutes to record post-fatigue tension.

Solutions

The muscles were immersed in normal Ginsborg saline[26,27] composed (in mM) of: 167 NaCl, 5 KCl, 2 $MgCl_2$, 5 $CaCl_2$, and 2 imidazole-chloride, with pH adjusted to 7.4. Finally, normal saline containing dextrose 2 g/L was added.

The solutions contained a specific inhibitor of the mitoK$_{ATP}$ channel (5-hydroxydecanoate, 500 µM; Sigma, St Louis, MO), the mitoK$_{ATP}$ channel activator, diazoxide (10, 30, and 100 µM; Sigma), a potent selective inhibitor of the cGMP-dependent protein kinase (KT5823, 1 µM; Sigma), a nitric synthase oxide protein inhibitor (Nω-Nitro-L-arginine methyl ester hydrochloride, L-NAME, 100 µM; Tocris, Bristol, UK), and a protein kinase C inhibitor (staurosporine, 1 µM; Calbiochem, La Jolla, CA). All probe solutions were prepared by adding the correct volume of a stock solution dissolved in water or in dimethylsulfoxide (0.1% v/v for diazoxide and staurosporine) to the saline bath. Solution exchange was carried out via a three-way tap located at one end of the central channel of the experimental chamber.

Statistical analysis

Analysis of the experimental data took into consideration the following twitch parameters: amplitude to twitch peak tension expressed in g (from the basal resting tension to the maximal tension), and total tension (area under the tension-time curve). The control twitch was used as 100% and the probe twitch was expressed as the percentage in relation to the control. Twitch was obtained by electrical stimulation (see above).

Tension was measured as the maximum tension from the basal line to the peak before, during, and after the addition of the experimental drug (peak tension), and the tension-time integral was obtained from the area under the twitch profile. We compared each experimental condition. Data analyses were carried out using the Clampfit subroutine of the pClamp 9.2 software (Axon Instruments) and graphs were elaborated using Sigmaplot 10.0 software (Systat Software Inc. Erkrath, Germany). Results were expressed as the mean ± standard error of the mean, followed by the n value. Comparison of means was done using the Student's *t*-test, accepting a significant effect when P was <0.05.

Results

Diazoxide increases twitch tension of fatigued slow skeletal muscle fibers

To determine the possible participation of mitoK$_{ATP}$ during post-fatigue tension in the slow skeletal muscle of the chicken, the effect of diazoxide (10, 30, and 100 µM), considered to be a selective activator of the mitoK$_{ATP}$ channel, was explored (Figure 1). Diazoxide at 30 µM strongly increased the tension of the fatigued muscle.

Figure 2 shows that basal tension (considered as 100%) was reduced to 33.71% ± 8.62% in peak tension during fatigue; nevertheless, in the presence of diazoxide 30 µM, tension was increased to 103.38% ± 7.97% indicating post-fatigue (peak tension). Figure 2B also shows the effect of diazoxide on total tension, which increased the post-fatigue tension to 163.82% ± 11.42% in relation to fatigued muscle tension (n = 4; $P < 0.05$). These results are consistent with previous reports in which diazoxide antagonizes fatigue.

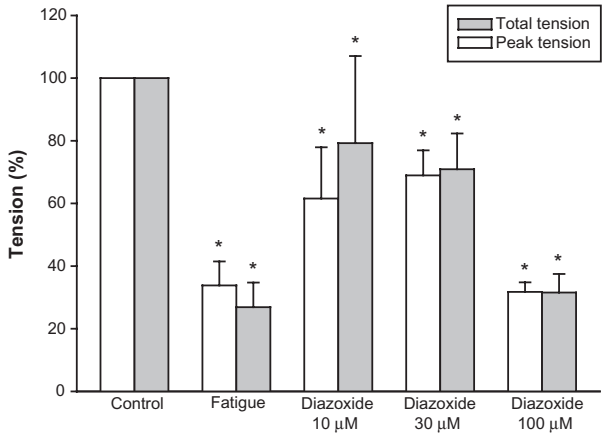

Figure 1 Normalized tension of the control twitch.
Notes: The third and fourth columns in the graph correspond to the peak and total tension of the twitch in fatigued muscle. The following columns show the post-fatigue effect of diazoxide (10, 30, and 100 µM). At 10 and 30 µM, diazoxide increased the post-fatigue tension in both peak tension and total tension. Normalization was done to compare tension in percentages for different bundles, given that each bundle possesses a different number of muscle fibers.

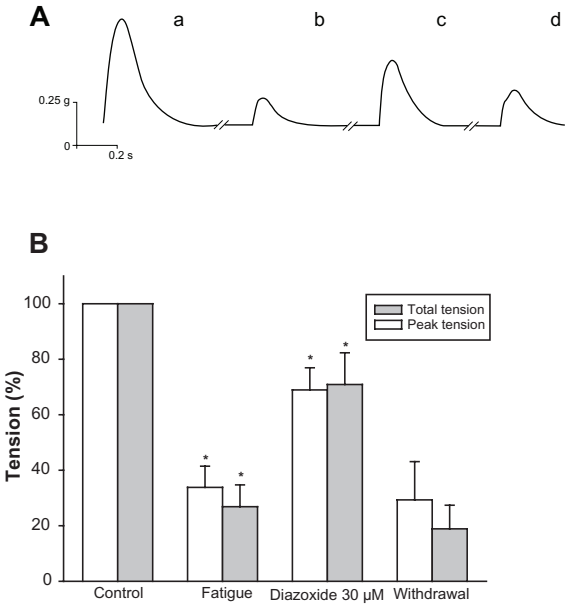

Figure 2 Effects of diazoxide 30 µM on twitch at almost 60% of muscle fatigue. (**A**) Representative recorded tension at the beginning of the experiment is represented by (**a**) In trace (**b**), the recorded tension approximately one hour after stimulating the bundle at 0.2 Hz is shown. Trace (**c**) displays the increase in the tension of the fatigued muscle 6 minutes after application of diazoxide. Trace (**d**) shows the tension recorded 10 minutes after withdrawal of diazoxide. (**B**) Effects of diazoxide on single twitch post-fatigue tension.
Notes: Diazoxide produced a significant increase in the total and maximal tension of twitches in fatigued muscle fibers (n = 4). *$P < 0.05$.

Effect of 5-hydroxydecanoate on twitch tension in fatigued slow skeletal muscle fibers

To determine the possible participation of mitoK$_{ATP}$ in post-fatigue tension, the action of the mitoK$_{ATP}$ channel-selective inhibitor, 5-hydroxydecanoate, was studied at the previously reported concentration range (500 μM).[22,28] The maximum tension obtained prior to adding the drug was the control tension (100%). A 50% reduction in control tension was considered to be fatigue. The 5-hydroxydecanoate was added once fatigue was reached, and tension was recovered at 11.91% ± 1.6% in relation to muscle fatigue tension. We observed a tension recovery (15.3% ± 4.8%) in the presence of 5-hydroxydecanoate. Nevertheless, this difference was not statistically significant (n = 4; $P > 0.05$).

Effect of diazoxide and 5-hydroxydecanoate on tension in slow muscle fibers

Previous reports have shown that diazoxide antagonizes fatigue by opening mitoK$_{ATP}$ channels, which was corroborated by adding 5-hydroxydecanoate and blocking the effect of diazoxide.[22] Figure 3 shows the results when diazoxide 30 μM and 5-hydroxydecanoate 500 μM were added. According to these results, there was an increase by 5.91% ± 4.99% with respect to control (standardized 100%). However, there was an increase of 26.0% ± 4.99% in peak tension, whereas the total tension increment was 44.87% ± 7.90% with respect to fatigue tension; nevertheless, the effect was statistically significant ($P = 0.025$ and $P = 0.023$, respectively). These results suggest that 5-hydroxydecanoate in combination with diazoxide acts as an agonist during fatigue, although it is possible that 5-hydroxydecanoate does not act as a selective inhibitor of mitoK$_{ATP}$ in the slow skeletal muscle fibers of the chicken, due to substrate conversion by β-oxidation.[29]

Effect of diazoxide and staurosporine on tension in slow muscle fibers

Previous studies have implicated protein kinase C in mitoK$_{ATP}$ channel function. García et al[22] suggested that diazoxide has an antagonistic effect on muscle fatigue through several signaling routes. We studied the action of staurosporine (1 μM), a selective inhibitor for protein kinase C, in combination with diazoxide. Diazoxide and staurosporine caused an increase in post-fatigue tension in relation to muscle fatigue tension when added to the bath (Figure 4). The maximum tension (control) dropped to 75.77% ± 2.47% in fatigued muscle. Nevertheless, there was an increase in peak tension of 120.75% ± 3.54% (post-fatigue) and in total tension of 158.82% ± 8.60% . Both of these increases were statistically significant ($P = 0.007$ and $P = 0.021$, respectively). These results differ from those previously reported by García et al,[22] in which the effect of

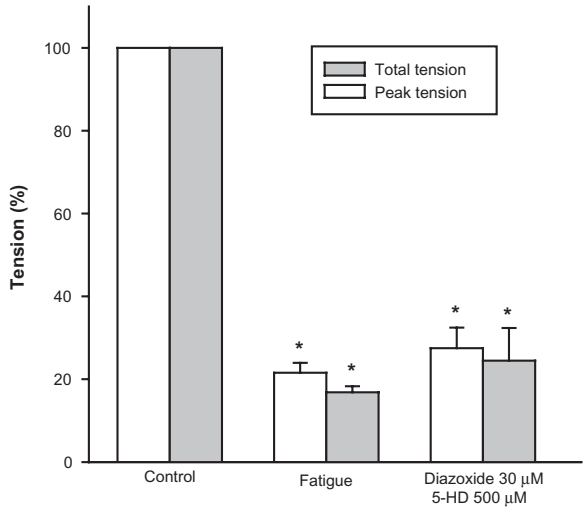

Figure 3 Effect of the combination of 5-hydroxydecanoate 500 μM and diazoxide 30 μM on the fatigued skeletal muscle tension, along with peak tension ($P = 0.023$) and total tension ($P < 0.05$).
Note: *Represents statistical significance. There is a significant increase in post-fatigue tension after application of both agents. First columns indicate controls standardized to 100%, second columns indicate a comparison of reduction of force from controls and third columns indicate the effect of used drugs compared against fatigue as 100%.

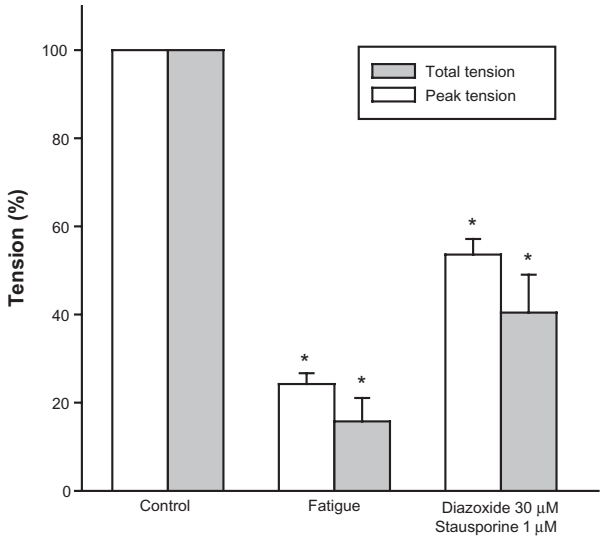

Figure 4 Effect of diazoxide and staurosporine on maximum and total tension.
Notes: *Represents statistical significance. The combination of drugs produced a great increase in post-fatigue tension in comparison with the tension of fatigued muscle without the drugs. Data correspond with those from other bundles following the same protocol. First columns indicate controls standarized to 100%, second columns indicate a comparison of reduction of force from controls and third columns indicate the effect of used drugs compared against fatigue.

diazoxide on post-fatigue tension was stronger when protein kinase C was blocked with chelerythrine and the two drugs were combined.

Effect of diazoxide and KT5823 on tension in slow muscle fibers

Previous reports have indicated that protein kinase G induces opening of the mitoK$_{ATP}$ channel in a manner similar to diazoxide. In addition, blockade of the channel by selective inhibition of protein kinase G with KT5823 has been reported.[19] To determine the possible participation of mitoK$_{ATP}$ during post-fatigue tension via this mechanism, KT5823 was used in combination with diazoxide. The combination of KT5823 1 μM with diazoxide resulted in an increase in post-fatigue tension with respect to the effect of diazoxide alone (Figure 5). The increment in maximal tension was 16.17% ± 6.04%, whereas the increase in total tension was 63.88% ± 4.74%. Both of these increases in tension were statistically significant ($P = 0.002$ and $P = 0.0001$, respectively). Our results show that KT5823 did not antagonize the effect of diazoxide, therefore promoting an increase in post-fatigue tension.

Effect of diazoxide and L-NAME on post-fatigue tension in slow muscle fibers

The effect of the combination of L-NAME, a selective nitric oxide synthase inhibitor, and diazoxide on mitoK$_{ATP}$ channels was investigated.[22] Figure 6 shows the effect of diazoxide 30 μM and L-NAME 100 μM on maximum and total tension. Comparing the average for muscle fatigue in relation to the control (100%), there was a decrease of 69.35% ± 5.18%, whereas the combination of diazoxide and L-NAME increased post-fatigue tension to 62.31% ± 12.38% ($P = 0.0003$) and produced a higher increase in total tension (91.22% ± 15.06% with respect to fatigue, $P = 0.0004$). García et al[22] reported inhibition when diazoxide plus L-NAME was present. However, our results showed just the opposite, ie, there was a remarkable increment in post-fatigue tension with a combination of the two drugs, and it was even higher with diazoxide alone.

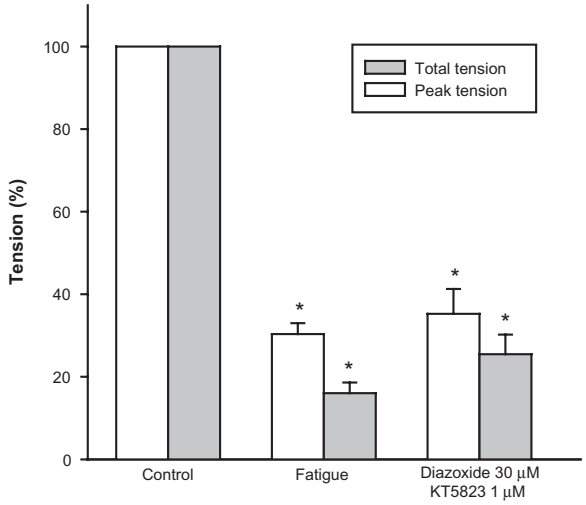

Figure 5 Effect of diazoxide and KT5823 on post-fatigued tension (maximum and total).

Notes: *Represents statistical significance. The combination of both drugs increased post-fatigue tension. Data correspond with those from the other bundles following the same protocol. First columns indicate controls standardized to 100%, second columns indicate a comparison of the reduction of the force respecting controls, and third columns indicate the effect of the used drugs compared against fatigue tension.

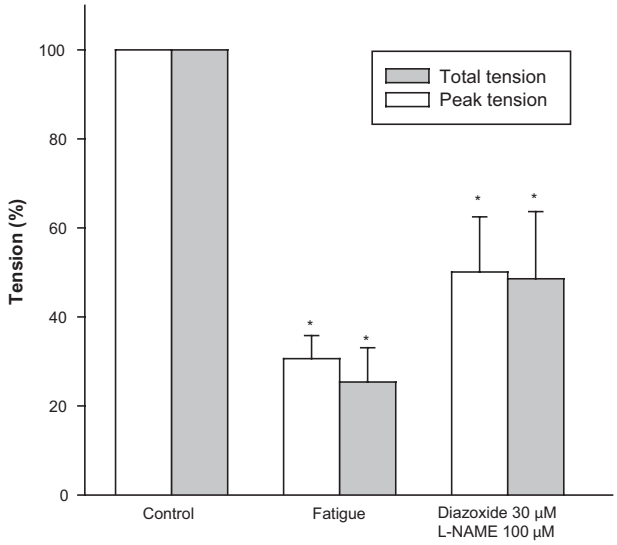

Figure 6 Effect of diazoxide and metil N^G-Nitro-L-arginine ester on the post-fatigue tension (maximal and total tension).

Note: *Represents statistical significance. The figure illustrates the tension twitches in different electrically stimulated bundles after the application of this drug combination, which significantly increased post-fatigue tension. First columns indicate controls standardized to 100%, second columns indicate a comparison of the reduction of the force respecting controls, and third columns indicate the effect of the used drugs compared against fatigue tension.

Discussion

The ADP/ATP relationship is a reflection of the metabolic necessities of the cell. Therefore, a decrease in ATP has an important role in the development of muscle fatigue, and any event that alters the production of this metabolite can determine the tendency of the muscle to become fatigued.[30]

In the present work, the effects of mitoK$_{ATP}$ channel agonists and antagonists were analyzed to clarify their possible participation during the fatigue process in slow skeletal muscle fibers of the chicken. Previously, García et al[22] reported activation of mitoK$_{ATP}$ during fatigue in fast ELD skeletal mammalian muscle. More recently, we suggested

the participation of K_{ATP} channels in avian muscle (anterior latissimus dorsi),[25] composed mainly of slow muscle fibers (type I).[31]

It has been reported that diazoxide 100 μM increased post-fatigue tension, and it was proposed that mitoK_{ATP} has a dominant role in this action. In the proposed analysis, diazoxide reduced fatigue without affecting twitches and/or tetanus, which suggests a direct influence on mitoK_{ATP}, discarding any effect on sarcolemmal K_{ATP} in addition to the specificity previously reported for this channel in isolated heart mitochondria.[13] It is important to point out that a diazoxide concentration was used and reported to have the maximum effects in these experiments.[28] In our results, diazoxide 30 μM had a statistically significant effect on post-fatigue tension (Figure 1), consistent with the results of García et al[22] but at a lower dose.

Activation of mitoK_{ATP} channels reconstituted in liposomes or in rat skeletal muscle bundles is inhibited by 5-hydroxydecanoate.[13,32] In contrast, we did not observe any effect on post-fatigue tension using 5-hydroxydecanoate 500 μM (data not shown). Perhaps this is because an intracellular mediator[13,29] or an activator could be required in order to guarantee mitoK_{ATP} activation.[22] Nevertheless, it is questionable to consider 5-hydroxydecanoate as a selective inhibitor because it is a potential metabolite for β-oxidation.[29] Recently, we have reported no modification in the respiratory rate of mitochondria from chicken skeletal muscle as a result of treatment with 5-hydroxydecanoate in relation to different respiratory substrates.[32] In this regard, Sarre et al[33] suggested the existence of other factors involved in the ischemic process implied during fatigue, upstream activation of protein kinase C, and inhibiting diazoxide activation of mitoK_{ATP} with chelerythrine. Costa et al[18] and Costa and Garlid[19] suggested that protein kinase G induces the opening of mitoK_{ATP} in a way similar to that achieved by the channel activators, diazoxide and cromakalim, in heart, liver, and brain mitochondria. Furthermore, they verified the suggested route through inhibition of channel opening by KT5823, a selective inhibitor of protein kinase G. García et al[22] proposed that protein kinase C and mitochondrial nitric oxide synthase act as intermediaries in the signaling route where mitoK_{ATP} is involved. Another proposed factor for mitoK_{ATP} activation is production of reactive oxygen species due to nitric oxide production and protein kinase C activation.

Our present results differ from those previously described for the heart and fast skeletal muscle of the rat. Nevertheless, it is important to mention that we worked with a different in vitro model of fatigue (avian slow skeletal muscle fibers) and with a different stimulation protocol (twitches). In our protocol, we successfully induced fatigue, and we considered that the fatigued muscle kept up its strength under ischemic conditions.[34,35] Protein kinase C has been reported to have a central role during ischemic preconditioning.[18]

According to previous studies, staurosporine inhibits the protective effect of ischemic preconditioning via protein kinase C inhibition in the rat heart.[36] Chelerythrine, a protein kinase C inhibitor, eliminates the inhibitory effect of diazoxide in fatigue.[22] In the present report, the combination of staurosporine with diazoxide showed an increase in post-fatigue tension (Figure 4). Simultaneously, we used a selective inhibitor of protein kinase G (KT5823) to confirm whether protein kinase G takes part in the upstream mitochondrial signaling route of mitoK_{ATP} through its phosphorylation.[18,19] Thus, we observed an increase in post-fatigue tension. These results do not concur with those reported for the rat heart (Figure 5). We suggest that KT5823 could not have effects on protein kinase G due to the experimental model (complete muscle) used. This is why it is necessary to perform experiments on single fibers and isolated mitochondria of skeletal muscle to confirm that KT5823 has an effect on protein kinase G in skeletal muscle.

In addition, the effects of the nitric oxide synthase inhibitor, L-NAME, were analyzed to determine whether mitoK_{ATP} channels are involved in the effect of diazoxide on the signaling cascade that involves nitric oxide. Nitric oxide, an important messenger molecule in cells, is formed during skeletal muscle activity[37] and regulates mitoK_{ATP} function.[28] L-NAME blocked the cardioprotective effects of diazoxide.[38] There is evidence indicating that nitric oxide directly activates mitoK_{ATP}.[21,39] García et al[22] maintain that a combination of diazoxide with L-NAME does not have any effect on post-fatigue tension, which could indicate that L-NAME does indeed inhibit fatigue. In our case, we observed an increase in the presence of both drugs. It is possible that mitoK_{ATP} could be activated by mitochondrial nitric oxide synthase,[33] a place the nitric oxide synthase inhibitor would not have access to.

In conclusion, the effects of K_{ATP} channel inhibitors on muscle fatigue are controversial. In some studies, no significant effects on fatigue have been found,[40,41] while others have reported increases in fatigue.[42] Further studies are needed in order to understand better the pharmacological properties of this type of channel in this type of tissue, because they could be the key to identifying the cellular mechanisms involved in slow muscle fiber fatigue. The importance of this study involves the use of a model of

fatigue composed of exclusively of slow muscle fibers, ie, the anterior latissimus dorsi of the chicken.

Acknowledgments

We wish to thank Humberto Cruzblanca for his kind donation of the staurosporine used in this study. ESD has a scholarship from CONACyT, México. This paper was partially financed by grant RMP-PROMEP-PTC311.

Disclosure

The authors declare they have no conflict of interests in this work.

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