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Original Research Article

Possible role of 18-kDa translocator protein (TSPO) in etifoxine-induced reduction of direct twitch responses in isolated rat nerve-skeletal muscle preparations

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Abstract

Purpose: *To determine the effects of etifoxine on directly-elicited twitch tension of isolated rat nerve-skeletal muscle preparations and to propose a possible explanation of the mechanism of the observed effect.*
Methods: *Striated muscles contractile activity was elicited by electrical field stimulation. The effects of etifoxine and nifedipine on direct single twitch response were studied.*
Results: *The results demonstrate that the effect of etifoxine on skeletal muscle depends on the concentrations: low concentrations (10^{-8} M and 10^{-7} M) have little effect on twitch tension, whereas higher concentrations (10^{-6} M and 10^{-5} M) induced a significant decrease in the direct single twitch response in comparison to controls. The mean IC_{50} (reduction of directly-elicited twitch tension) of etifoxine was 0.85 x 10^{-6} M. The selective L-type calcium channel blocker nifedipine (10^{-5} M) induced a greater decrease in the muscle force than 10^{-6} M etifoxine. The different abilities of etifoxine and nifedipine to reduce direct single twitch response may be related to their distinct mechanisms of action. The observed effect of etifoxine could be more complex. Probably etifoxine acts as a non-selective agent not only on L-type calcium channels $Ca_v1.1$ localized in sarcolemma but also on 18-kDa translocator protein (TSPO) in skeletal muscle.*
Conclusion: *Etifoxine-induced reduction of direct twitch responses could be attributed to an effect on TSPO and $Ca_v1.1$. Knowledge of the effects of TSPO ligands on the contraction of skeletal muscle might explain the role of TSPO in muscle contractility.*

Keywords: *Etifoxine, TSPO, Calcium channels, Direct single twitch response, Striated muscle*

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INTRODUCTION

Translocator protein (TSPO) is an 18-kDa ubiquitous protein and its primary intracellular location is the outer mitochondrial membrane [1,2]. The protein consists of a 169-amino acid sequence arranged as a five α-helical transmembrane structure [3]. Translocator protein is associated with some other proteins, e.g. the 32-kDa voltage-dependent anion

channel and the 30-kDa adenine nucleotide transporter. These three proteins are part of the mitochondrial permeability transition pore (MPTP) [4]. Different mitochondrial functions has been related to TSPO, including cholesterol transport and steroidogenesis [5], mitochondrial respiration, MPTP opening, regulation of the mitochondrial membrane potential, regulation of the mitochondrial respiratory chain, apoptosis and cell proliferation [5-10].

Translocator protein is also expressed in various other locations, apart from mitochondria. For example, TSPO is located on the outer cell membrane and in the cell nucleus [4,11]. The research on TSPO located in areas other than the outer mitochondrial membrane would be interesting, because, until now, it receives little attention.

Translocator protein is expressed in many organs, although secretory and glandular tissues appear to contain higher amount of this protein [1]; intermediate levels of TSPO are observed in renal and myocardial tissues and lower levels are found in the brain and liver [1,12].

Translocator protein is active in many other functions such as brain damage as a result of the activation of microglia, effects on the immune system and the host-defense response related to the phagocytes, ischemia, inflammation, responses to stress, influence on voltage-dependent calcium channels, involvement in cell growth and differentiation, and cancer cell proliferation [10].

Translocator protein is also expressed in skeletal muscle where high levels of TSPO mitochondrial RNA are recovered [12]. The protein has been detected in rat diaphragm [13,14]. High levels of TSPO were observed in the skeletal muscle of transgenic mice by immunohistochemical analysis [15]. It is supposed that TSPO plays a role in muscle contraction [16].

Etifoxine (ETX), a clinically approved drug for the treatment of anxiety disorders, has been identified as a synthetic TSPO ligand. The mechanism of action of ETX includes potentiation of $GABA_A$ receptor function in a direct allosteric manner, as well as by an indirect mechanism involving the activation of TSPO [17].

The aim of this study is to define the effects of the TSPO ligand etifoxine on directly-elicited twitch tension of isolated rat nerve-skeletal muscle preparations and to provide a possible explanation of the mechanism of the observed effect.

EXPERIMENTAL

Animals, tissue preparations, and procedure

The experiments were approved by the Bulgarian Food Safety Agency and the Ethics Committee of the Medical University of Plovdiv, Bulgaria (approval nos. 87/9.01.2014 and 5/29.09.2016, respectively). The study was performed in accordance with Basel Declaration [18] and ICLAS Ethical Guideline for Researchers [19].

Male Wistar rats weighing 160 - 200 g were euthanized by an overdose of ketamine 180 mg/kg bw and xylazine 15 mg/kg bw and the transversus abdominis muscles were isolated. Preparations were obtained according to a previously described experimental protocol [20]. The muscle strips were immersed and isometrically fixed in individual organ baths containing 15 mL modified Krebs' solution maintained at 35,5 ± 0,3 ºC and constantly aerated with 95 % O_2 and 5 % CO_2. The pH of the solution was kept at 7.28 ± 0.08. Preparations were put in the organ baths in a random manner.

Striated muscles contractile activity was elicited by electrical field stimulation (EFS). Two platinum electrodes were connected to an electronic stimulator (EFS - PZ03, C-optic, Bulgaria) and were placed on both sides of each strip. The initial muscle tension applied to achieve isometric recording was 7 mN.

In order to obtain a direct single twitch response, an electrical stimulus of 60 V supramaximal intensity, a frequency of 5 Hz and 500 µs square-wave pulse duration was used. The duration of the muscle stimulation was 3 s, followed by a 7 s pause. The mechanical responses produced by direct stimulation were recorded isometrically with a force transducer (TRI 201, LSi LETICA; Panlab S.L., Barcelona, Spain) and a computer-based system. This allowed the recording, archiving and statistical analysis of mechanical muscle activity as described previously [21]. The interval of discretization was 1 millisecond.

After an equilibration period of 20 min, direct muscle stimulation was applied and the twitch contractions were recorded. This muscle activity was stable in the absence of any drug and was used as control twitch contraction activity. After this, nifedipine and etifoxine were added separately to the organ baths.

Seven concentrations of etifoxine 10^{-8}, 10^{-7}, 10^{-6}, 3.10^{-6}, 5.10^{-6}, 7.10^{-6} and 10^{-5} M were studied, and a concentration-effect curve was obtained.

Initially, the lowest concentration was added and a five-minute record of the twitch contractions was registered. The strips were washed out with Krebs' solution (3 - 4 times) before adding a higher concentration of etifoxine.

Nifedipine was studied at a concentration of 10^{-5} M.

The effects on the contractile activity of the drugs and their concentrations were evaluated about 25 min after their addition to the bath. In all experiments, direct muscle contractions were elicited by EFS applied 12 min after adding of 10^{-5} M pipecuronium to each organ bath. Pipecuronium induces a neuromuscular blockade and the presence of neuromuscular blocking agent excludes any possible indirect (nerve) stimulation of the muscle tissue.

The IC_{50} of ETX was calculated as the concentration of the drug required to produce 50 % reduction in the force of the muscle twitches (the control twitch contraction activity was taken as 100 %).

The maximal duration of the experiments involving a single muscle strip was 45 min after the isolation.

Drugs and solutions

Etifoxine (Stresam®, Biocodex, Gentilly, France) was suspended in 0.9 % saline containing 0.1 % Tween 20. Nifedipine was purchased from Sigma. The solution of pipecuronium was prepared using Arduan® (Gedeon Richter, Hungary), diluted in 0.9 % saline.

The preparation solution had the following composition: Na^+ (143 mmol/L), K^+ (5.84 mmol/L), Ca^{2+} (3.7 mmol/L) and Cl^- (157 mmol/L).

Composition of Krebs' solution: Na^+ - 143 mmol/L; K^+ - 5.84 mmol/L; Ca^{2+} - 2.5 mmol/L; Mg^{2+} - 1.19 mmol/L; Cl^- - 133 mmol/L; HCO_3^- - 16.7 mmol/L; $H_2PO_4^-$ - 1.2 mmol/L and glucose – 11.5 mmol/L.

Statistical analysis

All data are presented as mean ± SEM. After verifying the normality of distribution by a - Smirnov test, it was confirmed that all results were normally distributed. One-way analysis of variance (ANOVA), followed by Bonferroni Multiple Comparison Test and paired samples T-test (with the aid of SPSS.17) were employed for statistical analysis. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Figure 1 represents the concentration-response curve (Hill curve) of etifoxine, fitted to the isometrically developed tension by means of a non-linear least mean square approximation.

The mean IC_{50} value of etifoxine-induced reduction of directly-elicited twitch tension was 0.85 x 10^{-6} M (Hill coefficient = 1.15; n = 15).

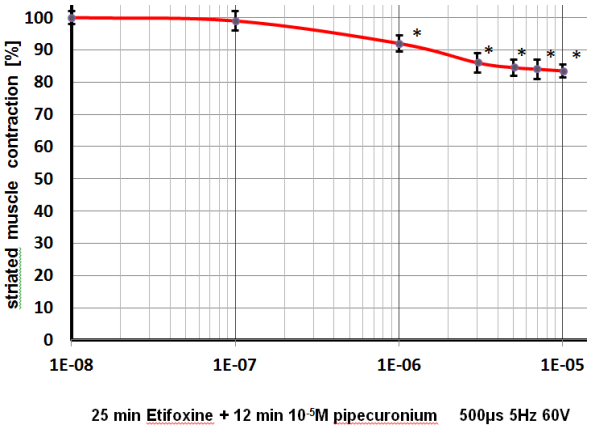

Figure 1: A concentration-response curve of 10^{-8} M ÷ 10^{-5} M etifoxine on the direct twitch responses of isolated rat abdominal striated muscle strips in the presence of 10^{-5} M pipecuronium. Twitch responses were evoked at 5 Hz; *$p < 0.05$ vs controls (One-way analysis of variance); n = 15

Etifoxine (concentration range 10^{-8} M ÷ 10^{-5} M) decreased the direct single twitch response in a concentration-dependent manner when 10^{-5} M pipecuronium was present in the organ baths. Low concentrations of ETX (10^{-8} and 10^{-7} M) reduced the muscle contractions evoked directly, but no statistically significant difference was found. However, etifoxine at concentrations equal to and higher than 10^{-6} M was more potent. In the presence of 10^{-5} M pipecuronium, a statistically significant decrease of the direct single twitch response was observed at 10^{-6} M etifoxine (5.3 ± 0.3 mN) and 10^{-5} M (4.7 ± 0.2 mN) in comparison to 10^{-8} M etifoxine (6.1 ± 0.3 mN), $p < 0.05$. Furthermore, the lowering of the muscle force was significantly greater with 10^{-6} M than that obtained with 10^{-7} M etifoxine (5.3 ± 0.3 mN vs 5.8 ± 0.3 mN, $p < 0.05$). The reduction of the direct twitch responses produced by 7.10^{-6} M (5.1 ± 0.4 mN) and 10^{-5} M (4.7 ± 0.2 mN) etifoxine was significantly higher ($p < 0.05$) than when etifoxine at a concentration of 10^{-8} M (6.1 ± 0.3 mN) and 10^{-7} M (5.8 ± 0.3 mN) was employed (Figure 1).

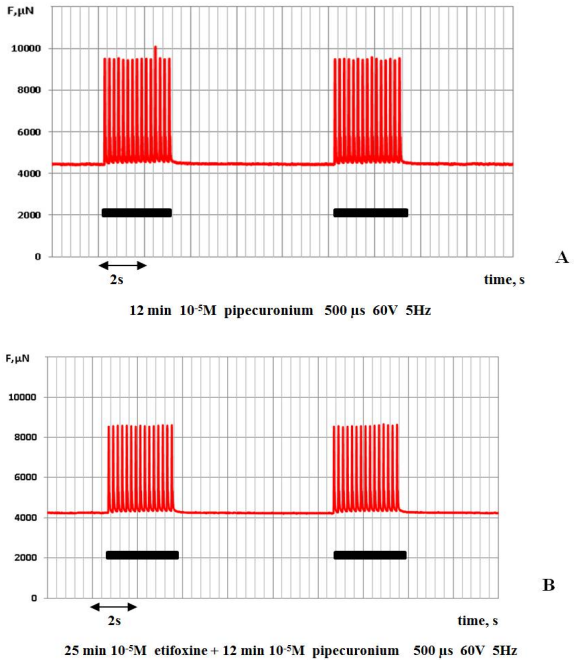

Figure 2: Effects of 10^{-5} M pipecuronium alone (A) and in combination with 10^{-5} M etifoxine (B) on direct muscle twitch tension of isolated rat abdominal (n. intercostalis - m. transversus abdominis) preparations. The direct twitch tension was evoked at 5 Hz and the duration of the stimulus is marked by ▬

As shown in Figure 2, etifoxine (10^{-5} M) was added to the organ baths about 13 min before addition of pipecuronium (10^{-5} M). Etifoxine at a concentration of 10^{-5} M evoked a significant reduction in the muscle force when compared to the controls (Figure 2).

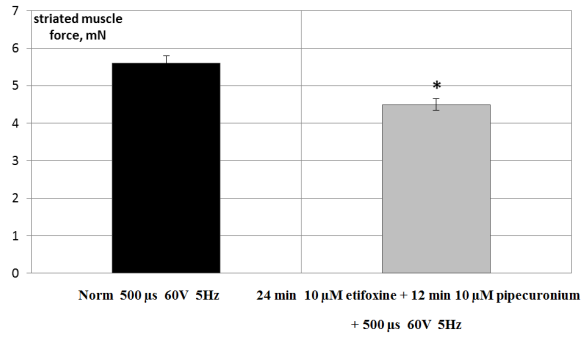

Figure 3: Effect of 10^{-5} M etifoxine on directly-elicited twitch tension of isolated rat abdominal striated muscle strips in the presence of 10^{-5} M pipecuronium. The direct twitch tension was evoked at 5 Hz; *p < 0.05 vs controls (paired T-test); n = 9

Etifoxine at a concentration of 10^{-5} M in the presence of 10^{-5} M pipecuronium had a significant effect on the directly-elicited twitch tension at 5 Hz and reduced the muscle force when compared to the controls (p < 0.05) (Figure 3).

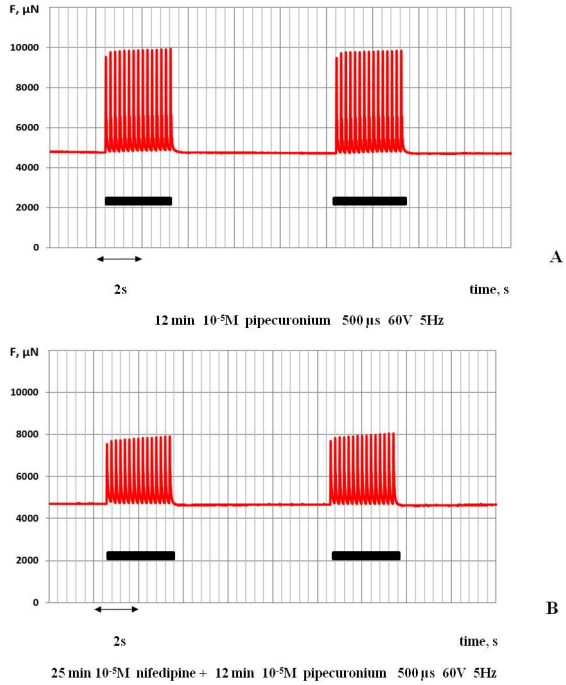

Figure 4: Effects of 10^{-5} M pipecuronium alone (A) and in combination with 10^{-5} M nifedipine (B) on the direct muscle twitch response of isolated rat abdominal (n. intercostalis - m. transversus abdominis) preparations. The frequency of the electrical stimulus was 5 Hz and the duration of the stimulus is marked by ▬

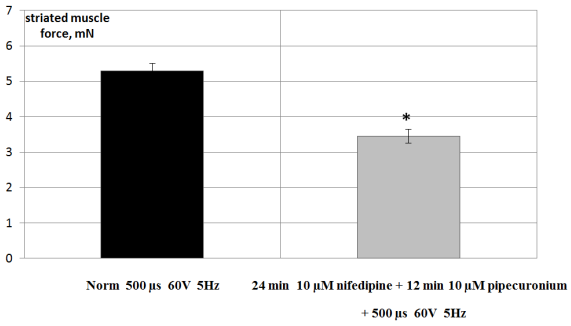

Figure 5: Effect of 10^{-5} M nifedipine on directly-elicited twitch tension of isolated rat abdominal striated muscle strips in the presence of 10^{-5} M pipecuronium. The direct twitch tension was evoked at 5 Hz. *p < 0.05 vs controls (paired T-test); n = 9

Figure 4 shows the effect of 10^{-5} M nifedipine on directly-elicited twitch tension. As expected, at this concentration nifedipine reduced the direct single twitch response significantly when compared to the control value in the presence of 10^{-5} M pipecuronium (3.5 ± 1.2 mN vs 5.8 ± 1.2 mN, p < 0.05) (Figure 5).

DISCUSSION

The results demonstrate that the effect of etifoxine on skeletal muscle was concentration

dependent: low concentrations (10^{-8} and 10^{-7} M) ETX have little effect on twitch tension, whereas higher concentrations (10^{-6} and 10^{-5} M) induce a statistically significant decrease of the direct single twitch response in comparison to the controls. The observed effects of ETX may have been due to the blockade of L-type calcium channels by the drug and/or binding to TSPO in the skeletal muscle.

The first hypothesis is that etifoxine blocks the L-type calcium channel, which leads to reduction in the force of the muscle twitches. To test this hypothesis, the effects of ETX were compared to these of nifedipine - a selective L-type calcium channel blocking agent.

The results show that nifedipine significantly reduces the direct single twitch response, suggesting a direct effect of the drug on skeletal muscle fiber (postjunctional action). The decrease in the directly elicited muscle contractions could be explained by the direct blockade of calcium channels in the skeletal muscle at the synaptic level.

The skeletal muscle contains calcium channels of the type $Ca_v1.1$ [22]. The $Ca_v1.1$ L-type calcium channels localized in the sarcolemma could be blocked by nifedipine. This process prevents calcium influx in the cytosol of the muscle cells and consequently reduces the muscle contraction. Nifedipine also acts as an antagonist of L-type calcium channels $Ca_v1.1$ localized in the transverse tubule membrane (aka DHPRs). However, the inhibition of DHPRs does not play a role in the reduced muscle contraction observed in presence of nifedipine.

The important structural and functional difference between the Ca^{2+} channel isoforms expressed in cardiac and skeletal muscle consists of a region that mediates the physical contact between DHPRs and ryanodine receptors (RyRs) in skeletal muscle.

Ryanodine receptors are a family of intracellular channels that play a role in the regulation of intracellular Ca^{2+} levels [23,24] and exist as three mammalian isoforms: RyR1, RyR2, and RyR3. RyR1 is the major isoform expressed in skeletal muscle and RyR2 in cardiac muscle. Furthermore, all three isoforms are expressed in the brain [24]. RyR1 and RyR2 play a critical role in Ca^{2+} release during excitation-contraction coupling [24], but the importance of RyR3 in mammalian tissue is not yet fully understood. The three isoforms differ in their sensitivity to activation and inactivation by Ca^{2+}.

RyR2 has the highest Ca^{2+} sensitivity [23,25,26] with reference to its function in releasing Ca^{2+} from cardiac sarcoplasmic reticulum by Ca^{2+}-induced Ca^{2+} release [25]. RyR1 is less sensitive to Ca^{2+} activation and inactivation since this isoform is coupled mechanically to L-type Ca^{2+} channels and it can be activated in the absence of extracellular Ca^{2+} [27]. RyR3 has the lowest Ca^{2+} sensitivity [26]. The mechanism of signal transduction between DHPR and RyR channels, which provides the coupling between the processes of excitation and contraction in the cardiac and skeletal muscle, is quite different [28].

In cardiac muscle, the depolarization of the cell membrane leads to opening of voltage-gated DHPR channel and subsequent Ca^{2+} influx. Thereafter Ca^{2+} binds to and activates the cardiac RyR2 isoform, which causes increased release of Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm, i.e. calcium-induced calcium release occurs. In cardiac muscle, DHPRs are randomly located and while DHPR and RyR2 are in close proximity to each other. A direct association or direct functional interaction between the two proteins could not be found. In cardiac cells extracellular Ca^{2+} should be present to provide the interaction between DHPR and RyR2 [28,29].

In skeletal muscle, the depolarization of the muscle cell membrane leads to a conformational change in a voltage-gated DHPR channel, which allosterically activates the skeletal RyR1 isoform. Hence, skeletal muscle contraction is induced by mechanical, direct physical interaction between DHPR and RyR1 and a subsequent release of Ca^{2+} from the sarcoplasmic reticulum into the cytosol (mechanical coupling).

In skeletal muscle, DHPRs are grouped into tetrads (groups of four receptors) and each DHPR is located immediately above one of the RyR1 subunits. These tetrads represent the structural link between DHPR and RyR1 that allows Ca^{2+} independent excitation-contraction coupling in skeletal muscle [28,29]. Thus, the inhibition of L-type calcium channels $Ca_v1.1$ localized in the sarcolemma, but not those localized in the transverse tubule membrane (DHPRs), is involved in the nifedipine-induced reduction of direct single twitch response.

The results show that, at equimolar concentration (10^{-5} M), nifedipine induces a greater decrease in the muscle force than etifoxine. It is possible that the different capacity of etifoxine and nifedipine to reduce direct single twitch response may be related to their distinct mechanisms of action.

The evidence suggests that etifoxine acts in a different way compared to nifedipine, which is a selective L-type calcium channel blocker. Thus, the nature of the observed effect of etifoxine could be more complex. Probably etifoxine acts as a non-selective agent not only on L-type calcium channels $Ca_v1.1$ localized in the sarcolemma but also on TSPO in skeletal muscle.

The second hypothesis regarding the influence of etifoxine on direct muscle contraction is the interaction with TSPO. Etifoxine activates the translocator protein 18-kDa (TSPO) [17]. Previous studies have shown that TSPO exists in the skeletal muscle [15], but its effect on the muscle contractility has not yet been fully explored.

Several studies have demonstrated that selective TSPO ligands significantly inhibit L-type Ca^{2+} channels in cardiac and vascular tissue [30,31]. Some potent and selective TSPO ligands interrupt the binding of (^{3}H)nitrendipine to L-type Ca^{2+} channels equally or even more effectively than the calcium antagonists verapamil and (+)-cis-diltiazem. In functional studies, one of these compounds (7-acetoxy-6-(*p*-methoxyphenyl) pyrrolo (2,1-*d*)-(1,5)benzothiazepine) shows selectivity for cardiac over vascular tissue.

This result represents a new implement about the role of TSPO in the cardiac function. Moreover, revealing the relationship between the chemical structure and the binding to TSPO and L-type Ca^{2+} channels could result in the synthesis of a new class of calcium channel blocking agents selective for cardiac tissue, with no affinity for TSPO [31].

The decrease in the muscle force observed in presence of etifoxine could not be explained only by influence on the calcium channels. It is possible that the reason for the reported difference in the effectiveness of etifoxine and nifedipine in reducing direct single twitch response is the further interaction of etifoxine with TSPO.

CONCLUSION

Etifoxine-induced reduction of direct twitch responses may be attributed to an effect on TSPO and L-type calcium channels $Ca_v1.1$ localized in the sarcolemma. Further experiments should be carried out to test this hypothesis. An understanding of the effects of TSPO ligands on the contraction of skeletal muscle would provide an understanding of the role of TSPO in muscle contractility.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Plamen Zagorchev conceived and designed the study, collected and analyzed the data, participated in the writing of the manuscript. Vesela Kokova designed the study, collected and analyzed the data, performed the statistical analysis and wrote the manuscript. Elisaveta Apostolova participated in the study design, the data collection, the statistical analysis and the writing of the manuscript. Lyudmil Peychev participated in the study design, the statistical analysis and supervised the study. All authors read the article and approved the final version of the manuscript.

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