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RAT SKELETAL MUSCLE CONTRACTILITY: THE ROLE OF BETA-ADRENOCEPTORS AND NITRIC OXIDE SYSTEM

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Both beta-adrenoceptors and the nitric oxide system play a significant role in the modulation of skeletal muscle contractility. Skeletal muscle adrenoceptors mainly belong to beta$_2$ subtype, while all three types of nitric oxide synthase may influence muscle contractility. The aim of our study was to investigate the possible interplay between beta-adrenoceptor agonists and nitric oxide system in the modulation of contractility of isolated rat hemidiaphragm. Adrenaline (0.05-1.5 μmol/L) and noradrenaline (1-5 μmol/L) given in a cumulative manner produced a concentration-related increase in Td. L-NAME (1, 3 and 10 mmol/L; 30 min of incubation) produced a significant, dose-dependent increase in Td of the muscle pretreated with cumulative concentrations of adrenaline (ΔTd up to 16%). When hemidiaphragm was pretreated with noradrenaline instead of adrenaline, L-NAME (3 mmol/L) it produced a similar potentiation of Td. In conclusion, nitric oxide seems to oppose beta-adrenoceptor potentiation of diaphragm contractility, and such an interaction depends on previous adrenoceptor stimulation. Nitric oxide probably decreases beta-adrenoceptor response via cGMP-induced stimulation of phosphodiesterase 2. The interaction between substances which modulate NO system activity and cAMP levels in the skeletal muscle may be of a great clinical importance for the treatment of certain respiratory and neurological diseases.

Key words: adrenaline, beta-adrenoceptors, L-NAME, nitric oxide, rat skeletal muscle contractility

INTRODUCTION

In contrast to a well known explanation of the role of beta-adrenoceptor in the heart, recently we began to understand more about the beta signaling pathway in skeletal muscles. Beta-adrenoceptors are involved in several muscle functions: oxygen consumption, glycogenolysis, lipolysis, ion exchange and

muscle contractility (Lynch and Ryall, 2008). Also, it was shown that some beta-adrenoceptor agonist have the ability to increase skeletal muscle mass and decrease body fat (Emery et al., 1984).

Numerous studies have reported that skeletal muscles contain a significant number of β-adrenoceptors. $Beta_2$-adrenoceptors are predominantly expressed in skeletal muscles from different species (Disatnik *et al*., 1990). Furthermore, Collet *et al*. (1998) have shown that only $beta_2$-adrenoceptors are expressed in the adult rat diaphragm. However, some authors found a significant population (≈20%) of β_1-adrenoceptors in the soleus muscle (Kim *et al*., 1991), as well as a smaller population of α adrenoceptors (Rattigan *et al*., 1986). Sillence *et al*. (1993) surmised the existence of atypical β_3-adrenoceptors in the rat soleus muscle. Also, Evans *et al*. (1999) have shown the existence of novel β_3-adrenoceptor subtypes (designated as β_{3a} and β_{3b}-adrenoceptors) (Evans *et al*., 1999). It is believed, that both β_3-adrenoceptor subtypes may exert stimulatory effects mediated through $G\alpha_s$ pathway. Furthermore, some authors (Hirata *et al*., 1986) observed the reduction in the number of sarcolemmal β-adrenoceptors and the occurrence of alpha-adrenoceptors in the rat soleus muscle, predominantly during hypokalemia.

There are two types of skeletal muscle fibres: type I fibres are designed for prolonged exertion (slow twitch fibres, *m. soleus*), and type II fibres are recruited during strenuous activities (fast twitch fibres, *m. extensor digitorum longus*). It has been documented that different types of skeletal muscle fibres express different densities and type of beta-adrenoceptors (Collet *et al*., 1998). The diaphragm is a muscle composed of an equivalent proportion of type I and type II fibres (Fratacci *et al*., 1996). It was shown that type I fibres contain a higher density of β-adrenoceptors (Martin *et al*., 1989).

The neuronal isoform of nitric oxide synthase (nNOS), most evident in fast fibres, has a major role in skeletal muscle contractility (Grozdanovic *et al*., 1997). It is documented that the activity of nNOS is regulated by calcium ions (Förstermann and Kleinert, 1995). The activity of nNOS can be upregulated during repetitive isometric contractions of skeletal muscles.

The aim of our study was to analyze the interaction between nitric oxide (NO) system and substances that modulate both the cAMP system and calcium metabolism in the skeletal muscle (e.g. adrenoceptor agonists), as well as the role of beta-adrenoceptors in such interactions.

MATERIAL AND METHODS

The experiments were performed on isolated rat hemidiaphragm. Wistar rats of both sexes (200-250 g) were bred and kept under standard laboratory conditions. The investigation conforms to the Guide For The Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1985). The hemidiaphragms from male and female rats were suspended in an isolated organ bath of 20 mL capacity. The muscle was immersed in Tyrode solution with a double amount of glucose (11.1 mM) and

bubbled with a mixture of 97% O_2 and 3% CO_2. The Tyrode solution composition was as follows: 136 mM NaCl, 2.81 mM KCl, 0.105 mM $MgCl_2$, 1.08 mM $CaCl_2$, 0.417 mM NaH_2PO_4, 11.9 mM $NaHCO_3$ and 11.1 mM dextrose. The temperature of the solution was 36°C. Two paladore wires were used to deliver the pulses for direct electrical stimulation. The diaphragm was secured to one of these wires at several points along the rib line. The other electrode was placed around the upper part of the diaphragm, but was not in contact with the muscle. The initial tension after 30-min equilibration period was about 5 g. The muscle was stimulated directly by subtetanic electrical stimulation. The frequency of stimulation was 14 Hz for 2 s, and a series of pulses was applied every 12 s (5 times/min). The isometric contractions were recorded with a microdisplacement myograph transducer (F 50, Narco-Bio-System, Inc; Houston, TX, USA) and recorded on paper (Physiograph IV polygraph). Values for the developed tension (Td) were obtained (for details, see Prostran and Varagić, 1986; Prostran *et al.*, 1993).

The effects of the used drugs were expressed as percentage changes of developed tension (Td) (ΔTd, %) in relation to the corresponding control. The experimental design is shown in Fig. 1.

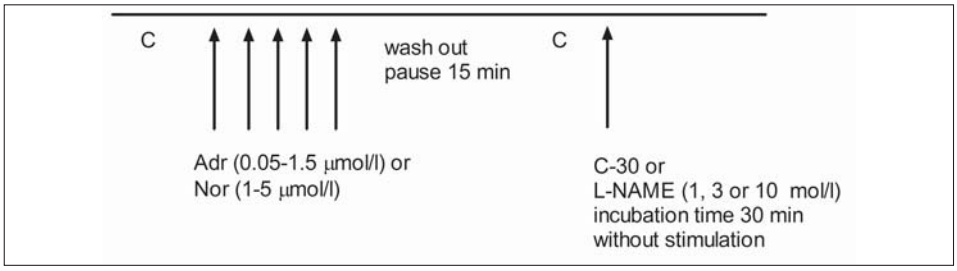

Figure 1. Experimental design.
C: the value of Td of the electrically stimulated muscle under basal conditions; C-30: the value of Td of the muscle, recorded after 30 min of incubation without L-NAME (Tyrode solution only), and without electrical stimulation; L-NAME: the value of Td of the electrically stimulated muscle recorded after 30 min of incubation with L-NAME (1, 3, or 10 mmol/L), as well as without electrical stimulation; Adr: adrenaline (cumulative concentrations of 0.05, 0.15, 0.35, 0.75, and 1.5 μmol/L); Nor: noradrenaline (cumulative concentrations of 1, 2, 3, 4, and 5 μmol/L).

The following drugs were used: L-noradrenaline bitartarate (Nor) (Serva, Heidelberg, Germany), epinephrine (Sigma Chemical Co., St. Louis, MO, USA), and N^G-nitro-L-arginine-methyl-ester (L-NAME, Sigma, St. Louis, MO, USA). Results are expressed as the mean ± S.E.M. of n determinations. Statistical analyses were performed with the software GraphPad Prism/Instat 1.1 (GraphPad Software; CA, USA) using nonlinear regression, one-way analysis of variance (ANOVA followed by post hoc 2-sided Dunnett's test), and Student's t-test, when appropriate. Values of $p<0.05$ were taken as statistically significant.

RESULTS

Adrenaline (Adr) given in a cumulative manner (0.05-1.5 μmol/L) produced a concentration-related increase inTd (ΔTd up to 33%; EC_{50} of 0.08 μmol/L, pD_2 of 7.08) (Fig. 2A).

Noradrenaline (Nor), also given in a cumulative manner (1-5 μmol/L) produced a concentration-related increase inTd (ΔTd up to 24%; EC_{50} of 1.11 μmol/L, pD_2 of 5.95) (Fig. 2B).

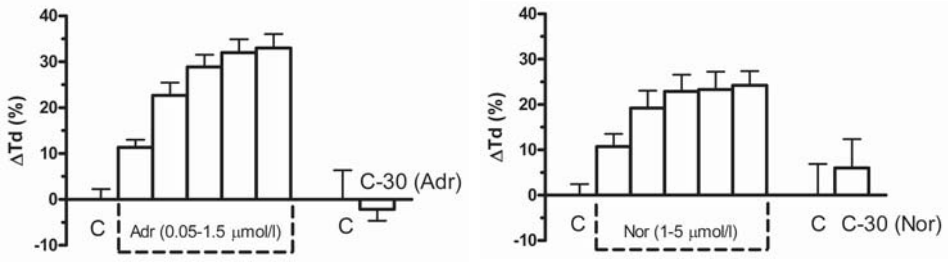

Figure 2. The effects of adrenaline and noradrenaline on Td of the isolated rat hemidiaphragm.
The effects of the repeated series of additions of adrenaline (Pannel A), and noradrenaline (Pannel B) on the isometric contraction of the isolated rat hemidiaphragm during direct subtetanic stimulation (Adr, 0.05-1.5 μmol/L; Nor, 1-5 μmol/L). ΔTd (%): the mean percentage change of Td from the corresponding control (C). C-30: the value of Td of the muscle recorded after 30 min of incubation without L-NAME (Tyrode solution only), and without electrical stimulation. Each vertical bar represents the mean + S.E.M. of 6-17 experiments

The next step was to investigate the effects of different concentrations of LNAME (1, 3, and 10 mmol/L) on the muscle pretreated with cumulative concentrations of Adr (0.5-1.5 μmol/L). It was found that L-NAME (30 min of incubation) produced a significant, dose-dependent increase in Td of the muscle pretreated with cumulative concentrations of Adr (ΔTd up to 16%; coefficient of determination, r^2 of 0.9990, $P = 0.02$) (Fig. 3, Pannels A and B).

In a separate series of experiments, the hemidiaphragm was pretreated with Nor (1-5 μmol/L) instead of Adr. Again, L-NAME (3 mmol/L, 30 min of incubation) increased ΔTd of the muscle pretreated with Nor. The effects of L-NAME (3 mmol/L) on ΔTd were similar with both types of pretreatment, Adr and Nor (C-30 vs. L-NAME of -2.40% vs. 4.40%, and 6.00% vs. 10.33%, respectively) (Fig. 3, Pannels C and D).

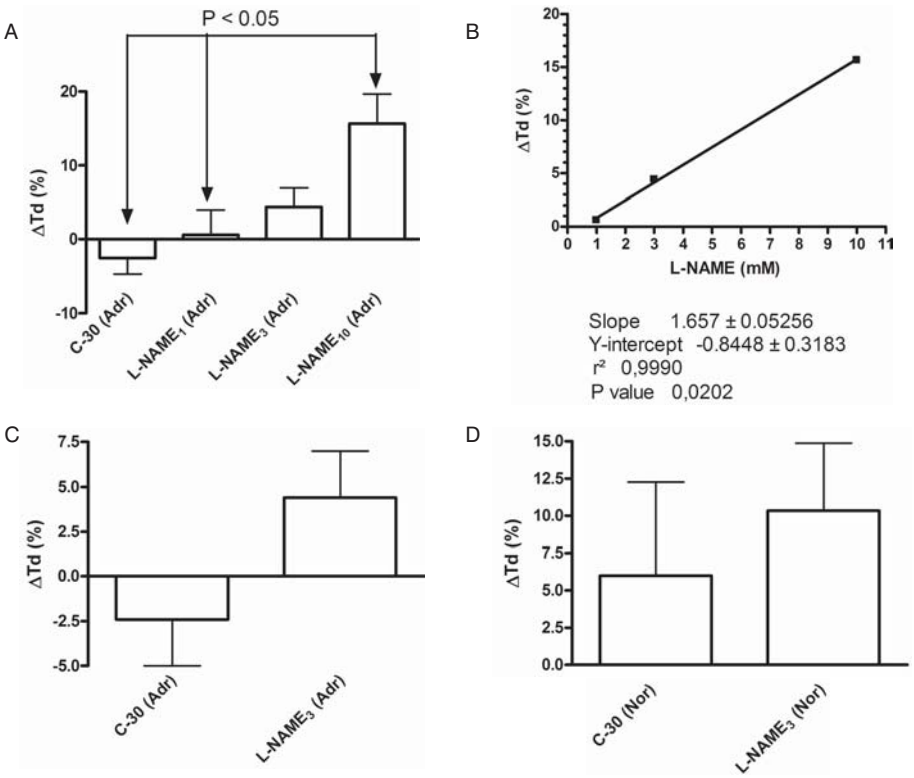

Figure 3. The interaction between adrenaline, noradrenaline and L-NAME on Td on the isolated rat hemidiaphragm.
Pannel A: Change of Td of the isolated rat hemidiaphragm pretreated with cumulative concentrations of adrenaline (0.05-1.5 μmol/L), during direct subtetanic electrical stimulation, after 30 min of incubation without or with L-NAME (1, 3 or 10 mmol/L). ΔTd (%): the mean percentage change of Td from the corresponding control (C). C-30 (Adr): ΔTd of the muscle pretreated with Adr (recorded after 30 min of incubation without L-NAME, and without electrical stimulation). L-NAME (Adr): ΔTd of the muscle pretreated with Adr (recorded after 30 min of incubation with L-NAME, 1-10 mmol/L, and without electrical stimulation). Each vertical bar represents the mean + S.E.M. of 5-6 separate experiments. $P<0.05$ (ANOVA with post-hoc 2-sided Bonferroni's test). Pannel B: the concentration-response regression line for the effects of L-NAME (1-10 mmol/L) on ΔTd (pretreatment with Adr). Each point represents the mean from 5-6 separate experiments. Pannel C and D: Changes of Td of the rat hemidiaphragm pretreated with cumulative concentrations of adrenaline (0.05-3 μmol/L) or noradrenaline (1-5 μmol/L), after 30 min of incubation without or with L-NAME, 3 mmol/L (C-30 and L-NAME, respectively). Ordinate: ΔTd (%): the mean percentage change of Td from the corresponding control (C). Each vertical bar represents the mean + S.E.M. from 5-6 separate experiments

DISCUSSION

Beta$_2$-adrenoceptor agonists, acting *via* cAMP, produce stimulation of fast skeletal muscle contraction. Activation of β_2-adrenoceptors leads to PKA-dependent phosphorylation of SR calcium release channel (ryanodine receptor) and transient increase in intracellular calcium concentration. These changes were observed in both fast-twitch and slow-twitch muscles. In contrast to the fast-twitch muscles, phosphorylation of the phospholamban, an inhibitory protein associated with the SR calcium uptake pump, can only occur in slow twitch muscles, leading to faster relaxation of this type of muscles.

Some results suggest the association between the type of skeletal muscle fiber, corresponding beta-adrenoceptors subtype and the pattern of regulation of muscle contraction *via* activation of beta-adrenoceptors. Despite coexistence of different β-adrenoceptors in the skeletal muscle, one receptor subpopulation could be more efficiently coupled to the response mechanism(s) than another. It was explained by specific second messenger allocation and intracellular compartmentalization (Kenakin, 1997; Alexander *et al*., 2009). Also, it was shown that β-adrenoceptors density is in correlation with the percentage of type I fibres in muscle (Collet *et al*., 1998), as well as with oxidative potential (Williams *et al*., 1984). Jensen *et al*. (2002) found that beta-adrenoceptor density correlated closely with the percentage of type-I fibres and inversely with the percentage of type-IIB fibres. In addition, Murphy *et al*. (1997) observed that chronic 14-day administration of a selective β_2-adrenoceptor antagonist (ICI 118551) resulted in an upregulation of β-adrenoceptors in the rat lateral gastrocnemius muscle.

Our results show for the first time that pretreatment with both adrenoceptor agonists may influence the NO-mediated decrease in stimulated diaphragm contractility. A non-selective NOS inhibitor L-NAME produced a dose-dependent increase in Td in pretreated muscle (Fig. 3, A and B). We have previously shown that NO could not influence diaphragm contractility under basal conditions (i.e., previously untreated muscle) (Stojanović et al., 2003).

In a previously published paper (Todorović *et al*., 2006), we tried to answer some questions regarding adrenoceptor control of the skeletal muscle contractility during different types of electrical stimulation: direct single pulse (DSPES) and subtetanic (DSTES). Electrical stimulation (ES) itself could induce a biphasic calcium response in skeletal muscle: DSPES mainly triggers a fast calcium signal sensitive to ryanodine and tetanic stimulation induces a long-lasting, inositol-triphosphate (IP3)-generated signal.

Noradrenaline in micromolar concentrations produced a concentration-related increase in Td despite the type of electrical stimulation. Atenolol, a selective β_1-adrenoceptor antagonist (10 μM, 10 min) did not change the response to noradrenaline regarding slopes of the curves and EC_{50} and Emax values during both types of ES. The obtained result is consistent with results of other authors who have not shown the expression of beta$_1$-adrenoceptors in the rat diaphragm (Collet *et al*., 1998).

On the other hand, ICI 118551, a selective β_2-adrenoceptor antagonist (0.01, 0.03, and 0.1 μmol/L; 10 min) decreased the maximal response to

noradrenaline, and at the highest concentration, it almost completely abolished the effect of noradrenaline on Td during DSPES. However, during DSTES, in the presence of ICI 118551, noradrenaline produced the maximal effect, but ICI 118551 significantly shifted noradrenaline curves to the right.

Based on these results, we concluded that the pattern of electrical stimulation of the muscle qualitatively changes the interaction between noradrenaline and β-adrenoceptor antagonists in the rat hemidiaphragm. In addition, β_2-adrenoceptors are probably involved in both types of electrical stimulation.

The neuronal isoform of nitric oxide synthase (nNOS), most evident in fast fibres, has a major role in skeletal muscle contractility (Grozdanovic *et al*., 1997). The activity of nNOS can be upregulated during repetitive isometric contractions of skeletal muscles. Therefore, the resting rat diaphragm produces approximately 3-5 pmol NO equivalents/mg/min of NO, while during active isometric contractions, diaphragm nitric oxide production is increased approximately sixfold (Stamler and Meissner, 2001). Nitric oxide can modulate skeletal muscle contractility *via* at least two mechanisms: through cGMP-NO pathway, and directly, by interacting with calcium release channels of the sarcoplasmic reticulum (Reid, 1998; Stojanović *et al*., 2004).

In the present experiments, L-NAME potentiated Td of muscle pretreated with either Adr or Nor (Fig. 2). As NO production was blocked by L-NAME, the amount of nitric oxide was not enough to activate solubile guanilate cyclase (sGC). Decreased cGMP-induced stimulation of phosphodiesterase 2 (PDE2) which leads to decreased degradation of cAMP (Wallis *et al*., 1999). A raised cytosolic concentration of cAMP facilitates phosphorylation of calcium-ion-conducting L-type channels and increases the likelihood of the channel being activated in response to stimulation. This mechanism could probably explain the potentiation of Td in the presence of L-NAME in muscle pretreated with beta-adrenoceptor agonists. It is already known that nitric oxide does not affect calcium current under basal conditions, but modulates sympathomymetic-activated L-calcium channels in cardiomyocytes (Brodde *et al*., 1999). It is quite possible to presume that such a mechanism exists in the skeletal muscle, as well.

Aminophylline (AMPh) modulates cAMP level by inhibiting phosphodiesterase (PDE) and produces an increase in the isometric contraction of the skeletal muscle. In the stimulatory action of AMPh on muscle contractility are probably involved both the enhanced calcium release from SR and the increased influx of extracellular calcium through sarcolemmal L-calcium channels (Prostran *et al*., 1993; Stojanović *et al*., 2004, 2005).

Under experimental conditions, during DSTES, L-NAME (non-selective inhibitor of NOS) increased tension developed (Td) in the muscle pretreated with cumulative concentrations of AMPh. As shown (Stojanović *et al.,* 2003), pretreatment of the muscle with cumulative concentrations of AMPh (0.36-3.60 mmol/L) could be a stimulus strong enough for upregulation of nNOS. Therefore, observed potentiation of muscle contractility is most likely due to specific blockade of nNOs (i.e. the loss of the inhibitory effects of nitric oxide on diaphragm contraction).

In previous studies, propranolol (1 μmol/L; 30 min incubation) did not change Td itself, but abolished the stimulatory effect of L-NAME in the rat hemidiaphragm (Stojanović *et al*., 2005). On the other hand, atenolol (1 μmol/L, 30 min incubation) neither significantly changed Td nor L-NAME-induced potentiation of Td.

CONCLUSION

In conclusion, pretreatment with adrenergic drugs may influence further response of the diaphragm to nitric oxide. Such an interaction may be of a great importance in the treatment of some respiratory and neurological disorders.

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KONTRAKTILNOST SKELETNOG MIŠIĆA PACOVA: ULOGA BETA ADRENERGIČKIH RECEPTORA I SISTEMA AZOT OKSIDA

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SADRŽAJ

Značajnu ulogu u modulaciji kontraktilnosti skeletnih mišića igraju i beta-adrenoceptori i sistem azot oksida. Adrenoceptori u skeletnom mišiću pretežno pripadaju $beta_2$ podtipu, dok sve tri izoforme sintaze azot oksida mogu uticati na kontraktilnost mišića. Cilj našeg istraživanja je bio da ispitamo eventualne interakcije između beta-agonista sistema azot oksida u modulaciji kontraktilnosti izolovane hemidijafragme pacova. Adrenalin (0,05-1,5 μmol/L) i noradrenalin (1-5 μmol/L), primenjeni kumulativno, dovode do koncentracijski-zavisnog porasta Td. L-NAME (1, 3 i 10 mmol/L; 30 min inkubacije) dovodi do značajnog, doznozavisnog porasta Td mišića koji je pretretiran kumulativnim koncentracijama adrenalina (ΔTd do 16%). Kada je hemidijafragma pretretirana noradrenalinom, a ne adrenalinom, L-NAME (3 mmol/L) je potencirao Td u sličnoj meri. Azot oksid izgleda smanjuje potenciranje kontraktilnosti izazvano aktivacijom beta-adrenoceptora, a ishod takve interakcije zavisi od prethodne stimulacije adrenoceptora. Azot oksid verovatno smanjuje efekte stimulacije beta-adrenoceptora putem cGMP-zavisne stimulacije fosfodiesteraze 2. Interakcija između supstanci koje modulišu sistem azot oksida i nivo cAMP-a u skeletnom mišiću može imati značajnu ulogu u lečenju određenih respiratornih i neuroloških oboljenja.