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RAPID COMMUNICATION

Different Sensitivity of Miniature Endplate Currents in Rat External and Internal Intercostal Muscles to the Acetylcholinesterase Inhibitor C-547 as Compared with Diaphragm and Extensor Digitorum Longus

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Summary

Derivative of 6-methyluracil, selective cholinesterase inhibitor C-547 potentiates miniature endplate currents (MEPCs) in rat external intercostal muscles (external ICM) more effectively than in internal intercostal muscles (internal ICM). Effect of the C-547 on intercostal muscles was compared with those on extensor digitorum longus (EDL) and diaphragm muscles. Half-effective concentrations for τ of MEPC decay arranged in increasing order were as follows: EDL, locomotor muscle, most sensitive = 1.3 nM, external ICM, inspiration muscle = 6.8 nM, diaphragm, main inspiration muscle = 28 nM, internal ICM, expiration muscle = 71 nM. External ICM might therefore be inhibited, similarly as the limb muscles, by nanomolar concentrations of the drug and do not participate in inspiration in the presence of the C-547. Moreover, internal ICM inhibition can hinder the expiration during exercise-induced fast breathing of C-547- treated experimental animals.

Key words

Miniature endplate current • Acetylcholinesterase • Anticholinesterase • Skeletal muscle

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Introduction

Some derivatives of 6-methyluracil inhibit the muscle acetylcholinesterase (AChE) in the different muscles with different efficacies (Reznik *et al.* 1998, Anikienko *et al.* 2008). These compounds inhibit acetylcholinesterase, which prolongs the decay time of the MEPC and increases the amplitude because the non-hydrolyzed ACh molecules diffuse from the cleft and can repetitively activate the receptor-channel complexes on the postsynaptic membrane. We found previously (Petrov *et al.* 2006) that the compound C-547 prolonged the duration of miniature endplate currents (MEPCs)(Giniatullin *et al.* 1989, 1993). In the soleus (slow locomotor) and extensor digitorum longus (fast locomotor) muscles, prolongation of the MEPCs was detected at nanomolar concentrations of C-547. In contrast, in the diaphragm muscle, an increase in the amplitudes of the MEPCs and the decay time constant appeared only when the concentration of C-547 was increased to $1x10^{-7}$ M. This explains why experimental animals treated with C-547 and other derivatives of 6-methyluracil survive easily even when their limb muscles were paralyzed. The effective concentrations necessary to paralyze the limb muscles while running on the treadmill

(EC_{50}) and those required for respiratory lethal failure (LC_{50}) differed significantly ($LC_{50}/EC_{50}>50$)(Zobov *et al.* 2004, 2005).

In the present work, we studied the effects of C-547 on the amplitudes and durations of MEPCs in two other important rat respiratory muscles- the external intercostal muscle (ICM) which contracts during inspiration and the internal ICM which is active during expiration (De Troyer 2005). The results were compared with data from the main respiratory muscle diaphragm and the fast locomotor *m. extensor digitorum longus* (EDL) which serves as a reference for hind limb muscles.

Methods

Experiments were performed on the isolated intercostals, EDL and diaphragm muscles excised from ether-anesthetized male Wistar rats (250-300 g body mass) in accordance with the ethical guidelines of the European Community for Animal Care and Exploitation. Both parasternal intercostal muscles of the fifth rib interspace, approximately 5 mm long, situated in the vicinity of the sternum, where inspiratory activity is greatest (De Troyer *et al.* 2005 for review) were dissected and pinned to the sylgard bottom of the superfusion chamber. Muscles were superfused at a rate of 2-3 ml/min with oxygenated Ringer-Krebs rat solution of the following composition (mM): NaCl 120.0, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, $NaHCO_3$ 11.0, NaH_2PO_4 1.0, glucose 11.0 pH 7.2-7.4 (Shabunova and Vyskočil, 1982, Urazaev *et al.* 1995). MEPCs were recorded in the synaptic zone by the standard two-microelectrode voltage clamp technique (3 M KCl, resistance 10-15 MΩ) at 20-22 ^{o}C. The membrane potential was held at -60 mV. At least 200 MEPCs were recorded in each fiber both before and for 30-60 min after pretreatment of muscles with C-547. They were digitized at 10 μs and analyzed using an original computer program for the amplitude, rise times (20-80 % of the maximal amplitude) and e-fold decay time constant (τ) (Bukharaeva *et al.* 2005, Samigullin *et al.* 2005, Volkov *et al.* 2007). Voltage-gated Na^+ channels were inhibited by adding 0.1 μM tetrodotoxin into the superfusing medium to increase threshold for muscle action potential (Vyskočil 1977) and prevent contractions after anti-AChE treatment when some large MEPCs could reach the spike threshold. Statistical analyses of electrophysiological data were performed using independent t-test ($p<0.01$) of Microcal Origin 6.0 program (OriginLab Corporation, Northampton, MA, USA).

1,3-bis[5(dietyl-o-nitrobenzylammonio)pentyl]-6-metyluracil (C-547, for its formula see Petrov *et al.* 2006, Fig. 1) was synthesized in Institute of Organic and Physical Chemistry of the Kazan Scientific Center of the Russian Academy of Sciences. Microelectrode glass, other drugs and salts were from Sigma (San Diego, CA, USA).

Results and Discussion

The application of C-547 in concentrations from 1×10^{-9} to 1×10^{-6} M increased the amplitudes and prolonged the durations of the MEPCs, as is typical for AChE inhibition (for averaged native records of MEPCs see Fig 1 inset B in Petrov *et al.* 2006). As reported before, the increases of the MEPC amplitude and decay time constant were significant already at nanomolar levels of C-547 in the EDL muscles. A concentration of C-547 of 5×10^{-9} M increased of the amplitude of the MEPCs in EDL by 60 %, and the duration of the falling phase (τ) by 250 % In contrast, in the diaphragm, significant increases in amplitudes (73 %) and decay time constants, τ (178 %) of the MEPCs appeared only when the concentration of C-547 was elevated to $1x10^{-7}$ M. From the complex curves on Figs 1A and 1B, the half-effective concentration (EC_{50}) for τ in the EDL was 1.3 nM and in the diaphragm it was 28 nM. In both muscles a further increase of C-547 concentration to 5×10^{-6} M reduced the amplitudes and the time constants of decay of the MEPCs (Figs 1A and 1B, right parts of the curves) (Svobodová *et al.* 2005).

In the intercostal muscles, different dose-response characteristics were found (Figs 1C and 1D). The MEPCs from inspiratory external ICM were prolonged much more by C-547 (EC_{50} for τ was 6.8 nM) than those from the expiratory internal ICM, where EC_{50} for τ was 71 nM. Internal ICM are therefore more than ten times less sensitive to the C-547 than external ICM and almost 70 times less sensitive than EDL.

Interestingly, the increase of τ of the MEPCs in external ICM was accompanied by a slight but significant increase of the MEPCs amplitude as expected, whereas virtually no increase of the amplitude was observed in internal ICM. This might indicate that the C-547 inhibits postsynaptic sensitivity in the internal ICM already in concentrations about 100 nM, whereas in other muscles the ACh receptors are less sensitive to antagonist-like effect of the C-547 as indicated by the amplitude of the MEPCs (Fig. 1. A, C, right part of the curves). However the difference in sensitivity of ACh receptors to the direct

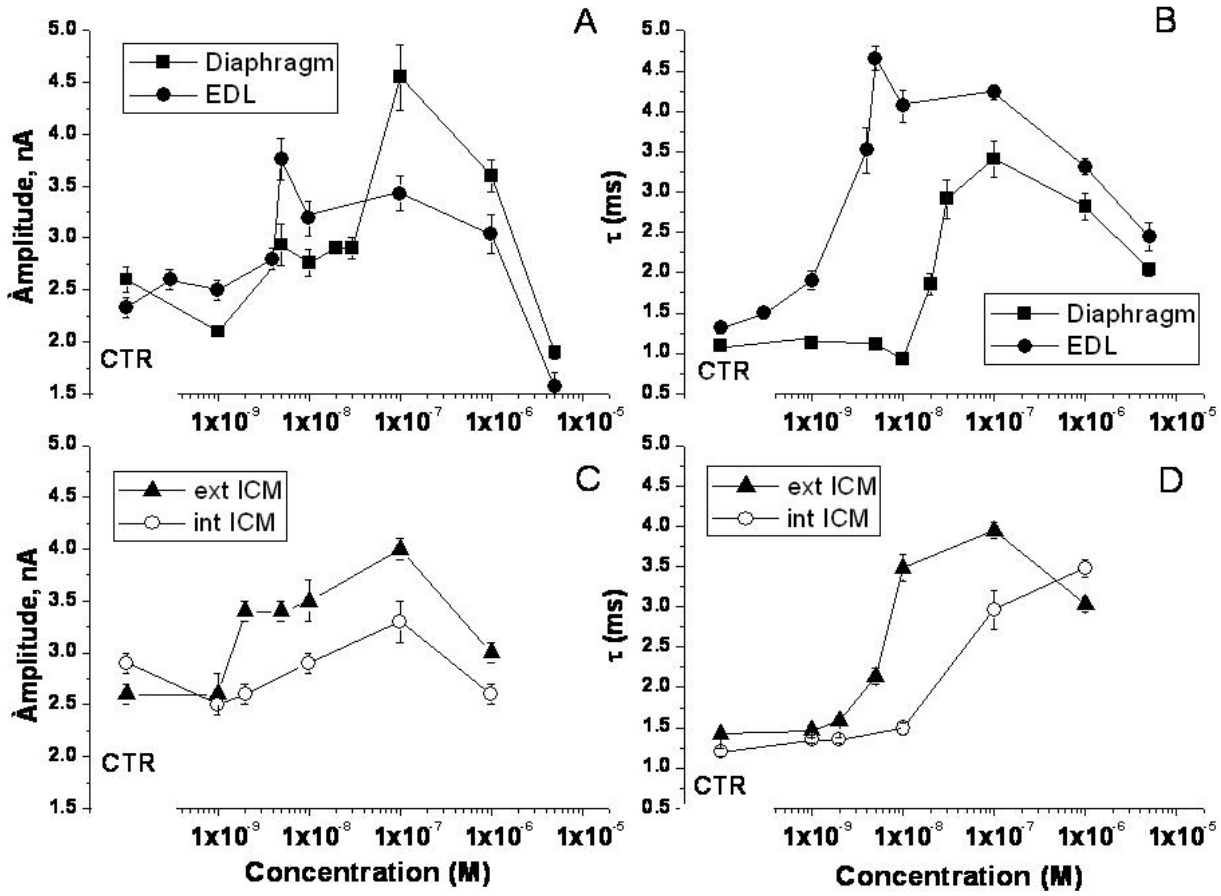

Fig. 1. Concentration-dependent effects of the C-547 on the amplitudes (A, C, in nA), and decay time constants (B, D, τ in ms) of miniature endplate currents (MEPCs) recorded (mean + S.E.M.) in 10 experiments from extensor digitorum longus (EDL) diaphragm (DIA), external (ex ICM) and internal intercostal muscles (int ICM). CTR – control before drug application. Note a significant increase of the decay time in EDL and inspiration ex ICM but not in diaphragm and in ICM after application of the C-547 in the concentrations of 5-10x10^{-9} M. Temperature 20 °C.

modulation by C- 547 compound in different muscles is not very probable as nicotinic receptors should be identical. Another possibility is that the absence of an increase in amplitude in the internal ICM is due to the backward presynaptic action of the quantal and/or non-quantal ACh (the latter is revealed in the presence of anti-AChE, Vyskočil *et al.* 1983, Doležal *et al.* 1983) on the number of acetylcholine molecules in the synaptic vesicle forming the single MEPC (quantal size, Van der Kloot 2003, Magazanik and Vyskočil 1969).

As already reported, experimental animals treated with C-547 survived even when their limb muscles were paralyzed. While it is known from our previous experiments (Petrov *et al.* 2006) that their breathing is unimpaired due to the 20-50 times lower sensitivity of the main respiratory muscle diaphragm to the C-547, the present results indicate that external ICM muscles might be inhibited, similarly as the limb muscles, by nanomolar concentrations of the drug; however they do not participate in inspiration. Internal ICM inhibition, on the other hand, can hinder the expiration in particular the fast breathing during exercise.

Half-effective concentrations for τ of MEPC decay arranged in increasing order were as follows: EDL, locomotor muscle, most sensitive, = 1.3 nM, external ICM, inspiration muscle = 6.8 nM, diaphragm, main inspiration muscle = 28 nM, internal ICM, expiration muscle = 71 nM. The differences in sensitivity among skeletal muscles should be analyzed in more details. There might be morphological obstructions in some synapses which can hinder the drug diffusion or the sensitivity to the drug might be determined by the prevailing isoform(s) of the AChE (Zwart *et al.* 2000). Some forms might have a higher affinity for the C-547 as it has been shown for intracellularly localized AChEs (G1 form) and the brain-localized G4 form in the case of other AChE inhibitors (Younkin *et al.* 1982, Rakonczay *et al.* 1994, Cho *et al.* 1994). According to our preliminary results

the C-547 does not inhibit butyrylcholinesterase (BuChE) and as there is more BuChE in the diaphragm than in locomotor muscles (Petrov *et al.* unpublished), this can at least partly explain the higher resistance of the diaphragm MEPCs towards the C-547.

Conflict of Interest

There is no conflict of interest.

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