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Different Sensitivity of Miniature Endplate Currents of the Rat Extensor Digitorum Longus, Soleus and Diaphragm Muscles to a Novel Acetylcholinesterase Inhibitor C-547

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Summary

A novel derivative of 6-methyluracil, C-547, increased the amplitude and prolonged the duration of miniature endplate currents (MEPCs) which is typical for acetylcholinesterase inhibition. In the soleus and extensor digitorum longus significant potentiation was detected at nanomolar concentrations. In contrast, in the diaphragm muscle, the increase in the amplitudes of the MEPCs and the decay time constant τ appeared only when the concentration of C-547 was elevated to $1x10^{-7}$ M. Possible consequences for the exploitation of this drug, which can selectively inhibit AChE in particular synapses, are discussed.

Key words

Miniature endplate current • Acetylcholinesterase • Anticholinesterase

Introduction

It has been demonstrated that some derivatives of 6-methyluracil are highly selective inhibitors of mammalian acetylcholinesterase (AChE) *in vitro* as shown by the values of the inhibitory constant, k^0, between $7.6x10^8$ and $3.5x10^9$ $M^{-1}min^{-1}$ for bovine erythrocytes (Reznik *et al.* 1998, Anikienko *et al.* 2001). During forced exercise experiments on dogs and rats these compounds displayed surprising and peculiar features. Animals treated with derivatives of 6-methyluracil survived easily even when their limb muscles were paralyzed. The concentrations required for paralysis of the limb muscles during functional loading while running on the treadmill (ED_{50}) and those required for respiratory failure (LD_{50}) differed significantly ($LD_{50}/ED_{50}>50$). This difference is much smaller for the common anticholinesterases (anti-AChEs) that are mostly organophosphorus inhibitors, carbamates and onium salts (Zobov *et al.* 2004, 2005). The difference for one

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6-methyluracil-based compound, 1,3-bis[5(dietyl-o-nitrobenzylammonio)pentyl]-6-metyluracil (laboratory code C-547, Fig. 1) was, in particular, quite large. It caused a distinct and prolonged (up to 7 days) relaxation of the locomotor muscles of dogs, rats and mice tested running on the treadmill, while the effects on respiration were minimal; the ratio LD_{50}/ED_{50} for C-547 reached 300 (Zobov *et al.* 2005).

The functional failure of motor muscles might be due to an accumulation of acetylcholine (ACh) in the synaptic cleft during high-frequency synaptic activity. This accumulation might result eventually in depolarization of the postsynaptic membrane and eventually to a failure to generate muscle action potentials (Hobbiger 1976, Vyskočil *et al.* 1983, Pope *et al.* 2005).

Therefore it was proposed that the different degree of relaxation of the locomotor muscles of animals treated with C-547 could be ascribed to differences in the ability of this compound to inhibit AChE in the endplates of limb muscles and in the diaphragm, which is the main mammalian respiratory muscle. To test this hypothesis, the direct estimation of the muscle AChE activity is of little relevance since it is known that the AChE localized in the synaptic area comprises only 10-20 % of the total AChE activity in muscles homogenates (Younkin *et al.* 1982). The use of intracellular microelectrodes to record miniature endplate current is more appropriate; it is based on the well-known fact that the non-hydrolyzed ACh molecules diffuse from the cleft and can activate the receptor-channel complexes on the postsynaptic membrane repetitively. This results in increased amplitude and prolonged duration of the postsynaptic potentials (or currents). This phenomenon is known as postsynaptic potentiation (Katz and Miledi 1973, Giniatullin *et al.* 1993).

In the present work we studied the effects of C-547 on the amplitudes and durations of miniature endplate currents (MEPCs) in isolated rat muscles of different functional types: fast locomotor – *m. extensor digitorum longus* (EDL), slow locomotor – *m. soleus* (SOL) and the diaphragm muscle.

Methods

Experiments were performed on the isolated nerve-muscle preparations of EDL, SOL, and diaphragm excised from the 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. Isolated muscles with 5-10 mm long nerve stump were placed into a chamber and 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. The pH was maintained at 7.2-7.4. MEPCs were recorded in the synaptic zone by the standard two-microelectrode voltage clamp technique (3 M KCl, resistances 10-15 MΩ) at 20-22 °C. The membrane potential was held at –60 mV. Amplified synaptic signals were digitized at 10 μs and at least 200 MEPCs were recorded in each fiber (n=10) both before and for 30-60 min after pretreatment of muscles with C-547. The MEPCs were analyzed using an original computer program for the amplitudes, rise times (20-80 % of the maximal amplitude) and e-fold decay time constants (τ) (Bukharaeva *et al.* 2005, Samigullin *et al.* 2005). Voltage-gated Na^+ channels were inhibited by adding 0.1 μM tetrodotoxin (Sigma, U.S.A) into the superfusing medium. This treatment prevented muscle contractions and displacement the inserted glass microelectrodes after anti-AChE treatment when some large MEPCs could reach the spike threshold. Statistical analyses of electrophysiological data were performed using independent t-test ($p<0.01$).

1,3-bis[5(dietyl-o-nitrobenzylammonio)pentyl]-6-metyluracil (C-547) was synthesized in the Institute of Organic and Physical Chemistry of the Kazan Scientific Center of the Russian Academy of Sciences.

Results and Discussion

The application of C-547 in concentrations from 1×10^{-9} to 5×10^{-6} M increased the amplitudes and prolonged the durations of the MEPCs, as is typical for AChE inhibition (Figs 1B and 1C). In the SOL and EDL muscles, the increases of the MEPC decay time constant were significant at nanomolar levels of C-547, i.e. 45 % and 69 %. The increases of the amplitudes of the MEPCs, 36 % in SOL and 61 % in EDL, and in the durations of the falling phase (τ increase by 156 % in SOL and by 256 % in ED) were observed at 5×10^{-9} M of C-547. 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 (Figs 1B and 1C). The maximum effects were seen at the same concentration. In all muscles a further increase of C-547 concentration to

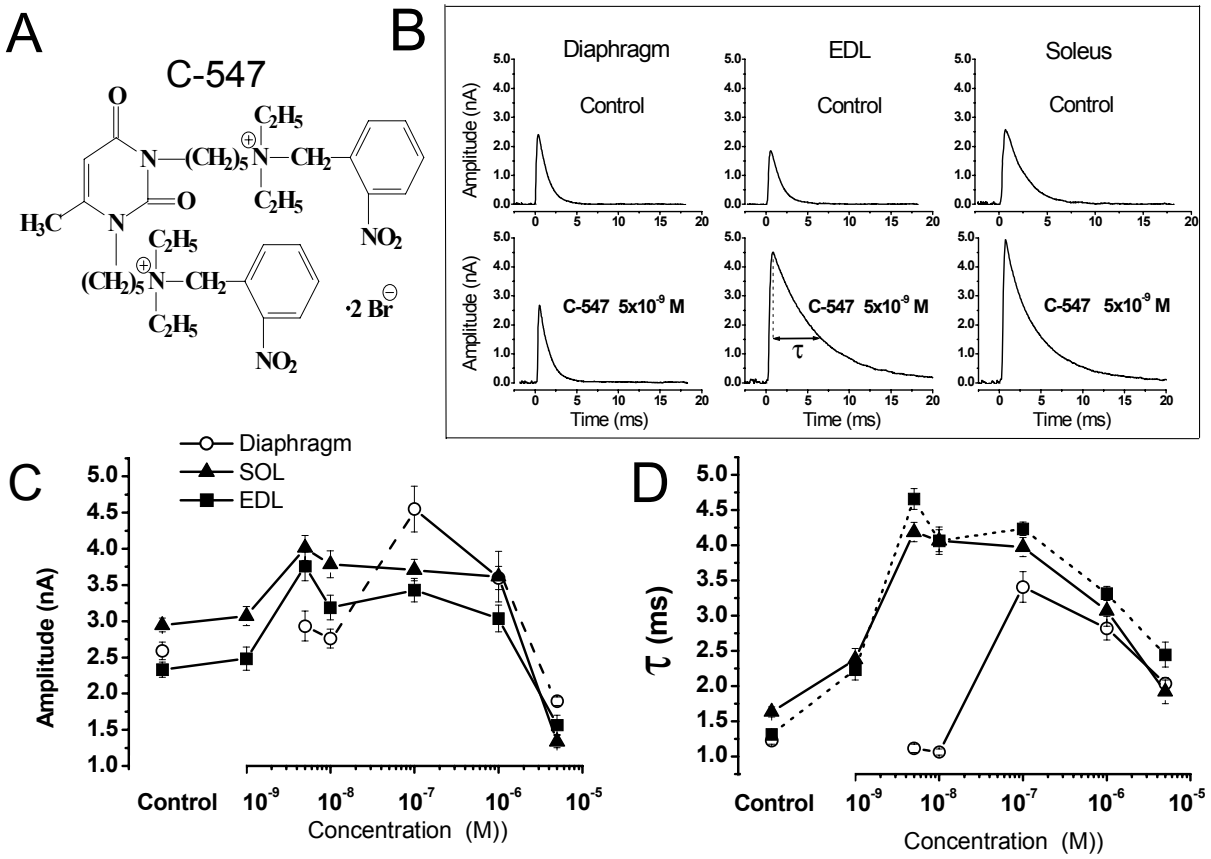

Fig. 1. Concentration-dependent effects of the C-547 (formula, A) on the amplitude (in nA, C) and decay time constant (τ in ms) of miniature endplate currents (MEPCs) recorded (mean ± S.E.M.) in 10 experiments (examples in inset B, 100 MEPCs averaged in each box) from diaphragm, extensor digitorum longus and soleus muscles. τ was estimated as time interval between 0.8 and 0.367 of MEPCs amplitude value (e-fold decrease, an example indicated by arrow in inset, B). Note a significant increase of the decay time in EDL and SOL but not in diaphragm after application of the $5x10^{-9}$ M C -547.

5×10^{-6} M reduced the amplitude and the time constant of decay of the MEPCs (Figs 1B and 1C, right parts of the curves). In addition to these changes, the presence of C-547 increased the rise times of the MEPCs. A significant prolongation of the rising phase of the MEPCs were first detected in the SOL (28 %) and EDL (46 %) muscles at a concentration 1×10^{-8} M (not indicated in Fig. 1). A similar increase (by 26 %) in the rise time in the diaphragm was noticed only with application of 1×10^{-7} M C-547. The maximal increases of the rise times in all muscles, i.e. in diaphragm, SOL and EDL were observed when the concentration of C-547 was raised to 5×10^{-6} M (corresponding to 101 %, 74 % and 115 % of control values).

The inhibitors of acetylcholinesterases are used in medical practice for the prolongation of the action of acetylcholine (ACh) in the central nerve system and periphery (Cummings 2000). However, because of the low tissue selectivity of the most common anticholinesterases, their use might be dangerous or could lead to death due to unexpected paralysis of the respiratory muscles during their administration (Pope *et al.* 2005). Moreover, organo-phosphorus insecticide self-poisoning is a major global health problem with hundreds of thousands of deaths each year (Eddleston *et al.* 2005). Thus there are good reasons to search for new, safer AChE inhibitors that might be active as myorelaxants in low doses and do not disrupt the vital functions of patients.

The present results demonstrate that the novel compound C-547 possesses many desirable properties. Its effect on the skeletal muscles can be ascribed to its anticholinesterase action, as demonstrated by the increase in the amplitude and the prolongation of action of single quanta at the motor endplate. The correlations between the AChE activity and MEPCs parameters have been analyzed in a number of studies, some of which used computer modeling. In particular, Anglister *et al.* (1994) showed that only a 6-fold decrease of density of the AChE active centers (from about 2500 μm^{-2} down to

400 μm^{-2}) causes the prolongation of falling phase of synaptic signals, whereas the amplitude of the signals depends almost linearly on the density of AChE active centers. The repetitive binding of ACh to the receptors and the resulting prolongation of decay would be greater if the anticholinesterase eliminated the greater number of cholinesterase active centers. In this respect, the observed changes of the amplitudes and durations of MEPCs are typical for substantial inhibition of synaptic AChE. However, quite atypical was the finding that the increases in amplitudes and prolongation of the time course were found in the hind limb muscle MEPCs (EDL and SOL) in concentrations of C-547 at least 20 times lower than those required in the diaphragm.

The lower sensitivity of the diaphragm might be a result of a difference in the ratio of the functionally active pools of butyryl- and acetylcholinesterase. If butyrylcholinesterase would play a more important role in the diaphragm than in SOL or EDL, the C-547 could be less potent, provided it does not inhibit the butyrylcholinesterase. However, to our preliminary experiments (data not given), the difference in the sensitivity to C-547 between SOL and EDL, on the one hand, and diaphragm, on the other hand, persists even after pretreatment of the muscles with the butyryl-cholinesterase-specific inhibitor 1×10^{-4} M iso-OMPA.

It should be noted that C-547, in higher concentrations (>$1x10^{-7}$ M, Fig. 1, panels C and D right), decreased the amplitude and shortened the falling phase of the MEPCs similarly in all three muscles. Such depression has been described for a number of anti-AChE drugs and explained either as a result of block of the open channels of the nicotinic receptor, competition with ACh for binding sites or from allosteric regulation of ACh receptor (Zwart *et al.* 2000, Krůšek and Vyskočil 2003, Krůšek *et al.* 2004, Svobodová *et al.* 2005). However, it is difficult to distinguish between these possibilities without further experiments with C-547, preferentially on whole cell patch recordings. What is clear, however, is that this additional effect cannot explain the selectivity of lower doses of the C-547 toward locomotor muscles.

The selective effect of the C-547 on MEPCs opens the door for checking its effect *in vitro* during repetitive high frequency stimulation and searching for drugs, which can selectively inhibit AChE in particular muscles.

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