BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01211.x
www.brjpharmacol.org

RESEARCH PAPER

Different sensitivities of rat skeletal muscles and brain to novel anti-cholinesterase agents, alkylammonium derivatives of 6-methyluracil (ADEMS)

Konstantin A Petrov[1,2], Lilia O Yagodina[2], Guzel R Valeeva[2], Natalya I Lannik[3], Alexandra D Nikitashina[2], Albert A Rizvanov[3,4,5], Vladimir V Zobov[1,3], Ellya A Bukharaeva[2,4], Vladimir S Reznik[1], Eugeny E Nikolsky[2,4] and František Vyskočil[6,7]

[1]*A.E.Arbusov Institute of Organic and Physical Chemistry, Russian Academy of Sciences, Kazan, Russia,* [2]*Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, Kazan, Russia,* [3]*Kazan (Volga Region) Federal University, Kazan, Russia,* [4]*Kazan State Medical University, Kazan, Russia,* [5]*Department of Genetics and Bioengineering, College of Engineering and Architecture, Yeditepe University, Istanbul, Turkey,* [6]*Department of Physiology, Faculty of Sciences, Charles University, Prague, Czech Republic, and* [7]*Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic*

Correspondence
Dr František Vyskočil, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14220 Prague, Czech Republic, E-mail: vyskocil@biomed.cas.cz

Keywords
acetylcholine; acetylcholinesterase; locomotor muscle; diaphragm; brain; alkylammonium anti-cholinesterases

Received
13 August 2010
Revised
24 November 2010
Accepted
5 December 2010

BACKGROUND AND PURPOSE

The rat respiratory muscle diaphragm has markedly lower sensitivity than the locomotor muscle extensor digitorum longus (EDL) to the new acetylcholinesterase (AChE) inhibitors, alkylammonium derivatives of 6-methyluracil (ADEMS). This study evaluated several possible reasons for differing sensitivity between the diaphragm and limb muscles and between the muscles and the brain.

EXPERIMENTAL APPROACH

Increased amplitude and prolonged decay time of miniature endplate currents were used to assess anti-cholinesterase activity in muscles. In hippocampal slices, induction of synchronous network activity was used to follow cholinesterase inhibition. The inhibitor sensitivities of purified AChE from the EDL and brain were also estimated.

KEY RESULTS

The intermuscular difference in sensitivity to ADEMS is partly explained caused by a higher level of mRNA and activity of 1,3-bis[5(diethyl-o-nitrobenzylammonium)pentyl]-6-methyluracildibromide (C-547)-resistant BuChE in the diaphragm. Moreover, diaphragm AChE was more than 20 times less sensitive to C-547 than that from the EDL. Sensitivity of the EDL to C-547 dramatically decreased after treadmill exercises that increased the amount of PRiMA AChE(G4), but not ColQ AChE(A12) molecular forms. The A12 form present in muscles appeared more sensitive to C-547. The main form of AChE in brain, PRiMA AChE(G4), was apparently less sensitive because brain cholinesterase activity was almost three orders of magnitude more resistant to C-547 than that of the EDL.

CONCLUSIONS AND IMPLICATIONS

Our findings suggest that ADEMS compounds could be used for the selective inhibition of AChEs and as potential therapeutic tools.

Abbreviations

AChE, acetylcholinesterase; ADEMS, alkylammonium derivatives of 6-methyluracil; BuChE, butyrylcholine esterase; ChE, cholinesterase; C-547, 1,3-bis[5(diethyl-o-nitrobenzylammonium)pentyl]-6-methyluracildibromide; EDL, extensor digitorum longus; iso-OMPA, tetra-isopropyl pyrophosphoramide; MEPCs, miniature endplate currents

Introduction

Acetylcholinesterase (AChE) ensures the functioning of cholinergic synapses by limiting the duration of ACh action. Partial inhibition of this enzyme is used in medical practice for the treatment of Alzheimer's and Parkinson's disease, traumatic brain injury and myasthenia gravis. However, virtually all anti-AChE agents possess various side effects mostly as a result of lack of selectivity among various organs and tissues (Caldwell, 2009). The drawbacks could be overcome by using inhibitors capable of inactivating AChE selectively. A new set of promising compounds, the alkylammonium derivatives of 6-methyluracil (ADEMS), have been recently synthesized and identified as inhibitors of AChE *in vitro* with inhibitory constants between 7×10^8 and 3×10^9 $M^{-1} \cdot min^{-1}$ (Anikienko *et al.*, 2008). During exercise of dogs and rats on the treadmill, these compounds displayed surprising effects. Animals treated with ADEMS had no breathing problems and easily survived even when their limb muscles were paralysed. In particular, one ADEMS compound 1,3-bis[5(diethyl-o-nitrobenzylammonium)pentyl]-6-methyluracildibromide (C-547) (Figure 1) was very effective in this respect. The concentrations required for paralysis of the limb muscles (ED_{50}) and those for respiratory failure (LD_{50}) differed significantly and the LD_{50}/ED_{50} ratio for C-547 was 300 (Kovyazina *et al.*, 2004; Zobov *et al.*, 2005).

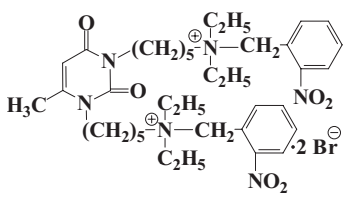
In an assay of single quantum miniature endplate currents (MEPC), anti-cholinesterase action is detectable as a prolongation of the MEPC decay phase. This postsynaptic potentiation is a consequence of the repetitive binding and activation of postsynaptic receptors by non-hydrolysed ACh and can be therefore used as a measure of the anti-cholinesterase activity (Giniatullin *et al.*, 1993; 2001; Kovyazina *et al.*, 2003). C-547 increased the amplitude and prolonged the time course of MEPCs at the rat locomotor muscles [specifically the extensor digitorum longus (EDL) and soleus muscles] even at nanomolar concentrations, whereas a 100-fold greater concentration was required to affect the MEPCs in the respiratory diaphragm and intercostal muscles (Petrov *et al.*, 2009).

The reasons for this differing sensitivity between the diaphragm and limb muscles are unknown. We therefore determined to what extent the anti-cholinesterase efficacy of ADEMS depends on the presence and position of 5(diethyl-o-nitrobenzylammonium)pentyl groups. For this question, three 6-methyluracil derivatives were compared in terms of their effect on MEPC. We also determined whether the difference in the sensitivity of the diaphragm and EDL to ADEMS compounds could be explained by the differing expression levels of butyrylcholine esterase (BuChE) in these muscles, as it was shown *in vitro* that some ADEMS inhibit BuChE at concentrations much higher than those inhibiting AChE (Anikienko *et al.*, 2008). The differences in sensitivity of the AChE itself to ADEMS between the diaphragm and EDL were assessed. Finally, we estimated the sensitivity to C-547 of purified AChE from the brain and EDL, after BuChE was inhibited.

Our results showed that the high intermuscular differences in the presence of ADEMS is partly caused by a higher level of ADEMS-resistant BuChE activity in the diaphragm and also by differing sensitivities of muscle and brain AChEs to ADEMS. Surprisingly, the brain AChE was almost three orders of magnitude less sensitive to C-547 than the AChE from leg muscles.

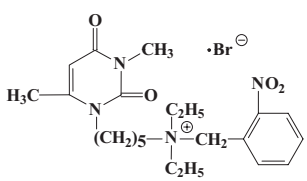
A. Compound C- 547

1,3-bis[5(diethyl-o-nitrobenzylammonium)pentyl] -6-methyluracil dibromide



B. Compound C- 627

1-[5(diethyl-o-nitrobenzylammonium)pentyl]-3,6-dimethyluracil bromide



C. Compound C-857

3-[5(diethyl-o-nitrobenzylammonium)pentyl]-1,6-dimethyluracil bromide

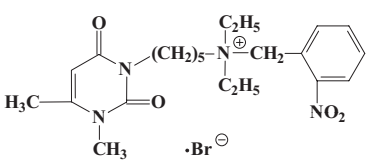


Figure 1
Structures of ADEMS compounds.

Methods

Animals

All animal care and experimental procedures were in accordance with the 'Guidelines for the Use of Animals in Neuroscience Research' by the Society for Neuroscience, guidelines of the European Communities Council and local guidelines approved by the Animal Ethics Committees of the Russian and Czech Academies of Sciences. We used muscles and brains excised from ether-anesthetized and decapitated Wistar rats of either sex (250–300 g body weight)

Treadmill exercise

Weight- and age-matched animals were randomly assigned to exercised (treadmill running) or control (cage-confined, sedentary) groups. The exercise protocol consisted of two sessions of enforced running (Columbus Instruments 4-channel rodent treadmill set at 9 $m \cdot min^{-1}$ and 5 degrees inclination) per day for 2 days. Daily sessions (alternating 10 min running, 5 min resting for a total of 90 min running per session) were separated by a 1 h 45 min rest period. Animals were killed and muscles quickly isolated for electrophysiology

approximately 18 h after the final fourth exercise session. No changes in body weight, muscle wet weight or total protein content were observed in animals subjected to the exercise protocols.

Endplate electrophysiology and electrophysiological criteria for inhibition of AChE

Isolated EDL and diaphragm muscles with nerve stumps were fixed in translucent chambers and superfused at a rate of 2–3 mL·min^{-1} with oxygenated Ringer-Krebs rat solution with 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 Vyskocil, 1982; Doležal *et al.*, 1983; Urazaev *et al.*, 1995). MEPCs were recorded in the synaptic zone using the standard two-microelectrode voltage clamp technique (2.5 M KCl, resistance 10–15 MΩ) at 20–22°C. The membrane potential was held at –60 mV. At least 200 MEPCs were recorded in each fibre before a 20 min bath application of either C-547, C-857, armin or paraoxon and then again after a 20 min washing with standard saline. Because neostigmine and C-627 are reversible, they were present in the muscle bath without washing. The MEPCs were digitized at 10 μs and analysed using an original computer program for the amplitude, rise times (20–80% of the maximal amplitude) and e-fold decay time constant (τ). Voltage-gated Na^+ channels were inhibited by adding 0.1 μM tetrodotoxin into the superfusing medium to increase the threshold for muscle action potential and prevent contractions after anti-AChE treatment, when some large MEPCs could reach the spike threshold (Lupa *et al.*, 1986). Anti-cholinesterase agents increase the amplitude and prolong the decay time of a single quantum MEPC, because non-hydrolysed ACh molecules diffuse from the cleft and can repetitively activate the receptor channel complexes on the postsynaptic membrane. This postsynaptic potentiation is shown in Figure 1 and was used here as a measure of anti-cholinesterase activity. As found previously (Petrov *et al.*, 2006), compound C-547 prolonged the duration of MEPC and the variable τ (decay time) was estimated as the time interval between 0.8 and 0.367 of the MEPC amplitude (e-fold decrease) An example of this is indicated by arrow in Figure 1, inset. Reliably assessing anti-AChE effects was based mostly on this increase in the τ of MEPC decay and not on the increase in MEPC amplitude (Land *et al.*, 1984), because the amplitude increase can be masked by the possible inhibition of postsynaptic receptors.

Hippocampal slice preparation, recording and data analysis

To assay the cholinergic induction of network oscillations, we used hippocampal slices prepared from 16–25 days old Wistar rats of both sexes. Animals were decapitated under deep ether anaesthesia and brains removed rapidly. Transverse slices (450 μm) were cut from the middle third of the hippocampus using a McIlwain Tissue Chopper (Surrey, UK). Slices were kept in an oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid of the following composition (in mM): NaCl 126, KCl 3.5, $CaCl_2$ 2.0, $MgCl_2$ 1.3, $NaHCO_3$ 25, NaH_2PO_4 1.2, and glucose 11 (pH 7.3) at room temperature (20–22°C) at least 1 h before use. Slices were placed into a conventional fully submerged chamber superfused with artificial cerebrospinal fluid (30–32°C) at a rate of 5–6 mL·min^{-1}. Extracellular field potentials were recorded using electrodes made from 50 μm diameter tungsten wire (California Fine Wire, Grover Beach, CA, USA). Electrodes were positioned in the CA3 subfield of the pyramidal cell layer and the signals were amplified using a custom-made amplifier (bandpass 0.1 Hz–3 kHz; ×1000). Recordings were online digitized (10 kHz) with a Digidata 1200 interface card (Axon Instruments, Union City, CA, USA) and analysed offline with the Axon package mini-Analysis (Synaptsoft, Decatur, GA, USA), and Origin 6.0 (Microcal Software, Northampton, MA, USA).

AChE and BuChE activity, AChE purification

Rat muscle homogenates were prepared in a Potter homogenizer with 0.05 M Tris-HCl, 1% Tween 20, 1 M NaCl, 2 mM EDTA; pH 7.0, at a ratio of 1:4 at 4°C. The activity of AChE and BuChE was measured according to Ellman *et al.* (1961). Activity was expressed in relation to the amount of total protein, which was determined by the Bradford colorimetric method, based upon binding of the dye Coomassie Brilliant Blue. When estimating the activity of AChE, 50 μM tetra-isopropyl pyrophosphoramide (iso-OMPA) (Koelle *et al.*, 1974) was added to inhibit BuChE. When estimating the activity of BuChE, 1×10^{-6} M [1,5-bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide] (BW284c51) (Atack *et al.*, 1989) was added to inhibit AChE.

Oligomeric forms of AChE were purified by ultracentrifugation at 120 000× *g* (Beckman Optima Max-E) for 4 h in a linear 5–20% sucrose gradient. Protein fractions were dialysed against 0.05 M potassium-phosphate buffer (pH 6.8) containing 0.5% Tween-20. After that, proteins were additionally purified using a Sepharose 6B 1.4 × 70 cm column and Logic System LP chromatography system (Bio-Rad, Hercules, CA, USA) at an elution rate of 20 mL·h^{-1}. Elution fractions with AChE activity were combined and used in further experiments. The purity of protein samples was assayed by Western blotting. Protein samples were separated using 10% SDS-PAGE gel electrophoresis and transferred to a poly(vinylidene fluoride) membrane. AChE was detected using specific anti-AChE affinity purified goat polyclonal antibody (E19, sc-6432, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Real-Time PCR

Total RNA from the diaphragm and EDL of three rats was isolated using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. cDNA synthesis was performed using a random hexamer primer and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT, Promega, Madison, WI, USA) at 37°C for 1 h. TaqMan primers and probes (Table 1) were designed using PrimerExpress software (Applied Biosystems, Foster City, CA, USA). Real-time PCR primers and TaqMan probes were synthesized by Syntol, Moscow, Russia. RealTime-PCR 2.5x premix (Syntol, Moscow, Russia) was used according to the manufacturer's instructions. The amount of RNA was normalized using β-actin as an internal control. TaqMan reactions were run in duplicate using a Bio-Rad iQ4 Multicolor

Table 1
Primers and probes for real-time PCR

Name	Forward primer	Reverse primer	TaqMan probe*
rmACTB	CGTGAAAAGATGACCCAGATCA	CACAGCCTGGATGGCTACGT	TGAGACCTTCAACACCCCAGCCATG
rBCHE	CCAACAGTGGATGGCGATTT	TCTGAGCTGTTTTCACTTTTCCAA	CTCACTGATATGCCCCACACACTACTCCAA
rAChE-Exon2	AACTACCGAGTGGGAACCTTTG	TGCGATATTTTCTTGTACCCATTG	TGTAGGCCTGCTGGATCAACGGCT
rAChE-Exon4″	TGCCGCGCAGCAATAC**	CCAACCCTTGCCGCC	CAGACCTGCGCCTTCTGGAATCGT**

*TaqMan probes contain 5′ FAM fluorescent dye and 3′ RTQ-1 quencher.
**For rAChE Exon 4″ detection, forward primer and probe for Exon 4 was used in combination with Exon 4″ specific reverse primer.

Real-Time iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The level of expression in the diaphragm was considered to be 100%.

Data analysis

Statistical analyses were performed using the independent Student *t*-test ($P < 0.01$) of the program Microcal Origin 6.0 (OriginLab Corporation, Northampton, MA, USA). Data are expressed as mean ± SEM. Differences between two means were determined using Student's *t*-test. Statistical significance was tested at 95% ($P < 0.05$) confidence level.

Materials

The following compounds were used: 1,3-bis[5(diethyl-o-nitrobenzylammonium)pentyl]-6-methyluracildibromide (C-547), 1-[5(diethyl-o-nitrobenzylammonium)pentyl]-3,6-dimethyluracilbromide (C-627), 3-[5(diethyl-o-nitrobenzylammonium)pentyl]-1,6-dimethyluracilbromide (C-857) (Figure 1) and armin [O,O-diethoxy O-(4-nitrophenyl) phosphate)] were synthesized in the A. E. Arbuzov Institute of Organic and Physical Chemistry of the Kazan Scientific Center of the Russian Academy of Sciences, Kazan. All other chemicals were purchased from Sigma (San Diego, CA, USA).

Results

Effects of alkylammonium derivates and other anti-cholinesterase agents on amplitude and decay time of MEPC in diaphragm and EDL

Alkylammonium derivatives. The application of C-547 in concentrations from 1×10^{-9} to 5×10^{-6} M increased the amplitude and prolonged the duration of the MEPC, as expected for AChE inhibition (Figure 2A, B and inset). In the EDL muscle, the increase of the MEPC decay time constant τ was significant at nanomolar levels of C-547, in accord with previous observations (Petrov *et al*., 2006; 2009). In contrast, in the diaphragm, substantial increases in amplitude (73%) and decay time constant, τ (178%) of the MEPC appeared only when the concentration of C-547 was raised to 1×10^{-7} M. Half-effective concentrations (EC_{50}) for τ of MEPC decay were 1.2 nM for the locomotor muscle EDL and 30 nM for the main inspiratory muscle from the diaphragm.

To determine whether the different sensitivities of the MEPC in the diaphragm and EDL towards C-547 was restricted to only this compound, the effects of two homologues of C-547 on the amplitude and decay time parameters of the MEPC in the diaphragm and EDL muscles were examined (Figure 3). These isomeric compounds designated C-857 and C-627 contain only one quaternary alkylammonium group in position 1 or 3 of the uracil cycle, respectively, instead of the two quaternary groups present in the C-547 molecule (Figure 1).

In contrast to C-547, where a potentiation of the MEPC amplitude was observed (Figure 2A), both C-857 and C-627 decreased the MEPC amplitude to about half of the control in the EDL as well as in diaphragm endplates starting from very low concentrations of 2–10 nM (Figure 3A and C). When concentrations above 10 nM were applied, there was a tendency to recovery, possibly because the anti-AChE effect overcame the postsynaptic cholinolytic effect.

Both C-857 and C-627 produced an increase in the MEPC decay constant τ, a change characteristic of anticholinesterase action, with differing efficacies in the diaphragm and EDL (Figure 3). The concentration of C-857 that brought about a 50% increase in τ (EC_{50} of decay prolongation) was 4.5 nM for the EDL and 380 nM for the diaphragm (Figure 3B).

The second compound tested, C-627, which can inhibit isolated AChE and BuChE almost equally (Anikienko *et al*., 2008), also increased the MEPC decay time constant τ in the EDL and diaphragm, starting from as low a concentration as 2 nM. In contrast to C-857, the EC_{50} of C-627 that produced a 50% effect in both muscles differed much less, by only a factor of five as it was 11 nM for the EDL and 55 nM for the diaphragm (Figure 3D).

Neostigmine, paraoxon and armin

Such a selective effect on the EDL and diaphragm was not observed when the effects of three standard anticholinesterase agents, namely the carbamate, neostigmine (Kuba and Tomita, 1971) and two irreversible organophosphorus inhibitors, paraoxon (Laskowski and Dettbarn, 1979; Vyskocil *et al*., 1983) [O,O-dimethyl O-(4-nitrophenyl) phosphate] and armin [O,O-diethoxy O-(4-nitrophenyl) phosphate)] (Nikolsky *et al*., 1992), were examined on the synaptic currents in the diaphragm and EDL. In contrast to ADEMS, neostigmine increased the MEPC amplitude and decay time in the EDL and diaphragm in the same concentration-dependent manner (Figure 2C and D). In other words, with

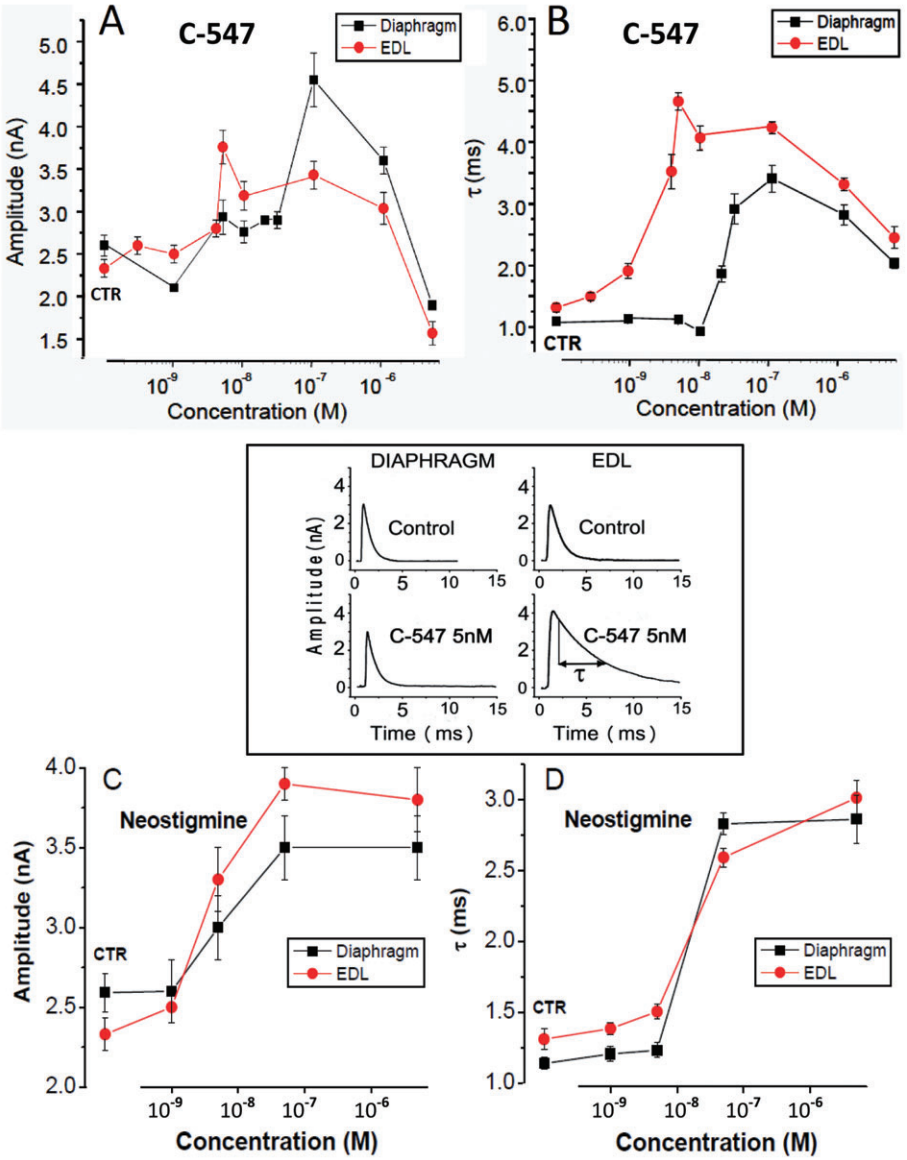


Figure 2

Concentration-dependent effects of C-547 (A, B) and neostigmine (C, D) on amplitude (in nA; A, C) and decay time constant (τ in ms; B, D) of miniature endplate currents (MEPC) recorded in 10 experiments from diaphragm and extensor digitorum longus (EDL) muscles. CTR, control values. Note the significant 4.5 times increase in decay time in the EDL but not in the diaphragm after the application of 5×10^{-9} M C -547 (B) and equivalent dose–response curves for neostigmine. Inset, time course of MEPC in single muscle fibre of diaphragm and EDL. For each record, 200 individual MEPC were pooled. While 5 nM of C-547 did not affect MEPC in the diaphragm, the drug increased the amplitude and τ (arrow) of the MEPC in EDL. This increase is typical when AChE is inhibited.

neostigmine there was no particular concentration that would be effective in the EDL and ineffective in the diaphragm. A similar outcome was obtained from results with paraoxon and armin. Armin also increased the MEPC amplitude and decay time in the EDL and diaphragm in the same concentration-dependent manner with maximum at 1 μM (amplitude) and 10 μM (τ of decay). In the case of paraoxon, the rate of increase of the τ in both muscles was measured after application of the drug in the concentration of 1 μM. The final prolongation of the MEPC decay by the factor of 3.7 was reached with the similar rate in the diaphragm and EDL. Half-times of τ increase was 23 ± 2 min (n = 5) and 22 ± 3 min (n = 5), respectively.

AChE to BuChE ratio and gene expression in diaphragm and EDL

The high intermuscular differences with C-857 and C-547 (neither drug inhibited BuChE *in vitro*) and low intermuscular difference in their sensitivity to C-627 (which inhibits BuChE and AChE almost equally) (Anikienko *et al.*, 2008) suggested

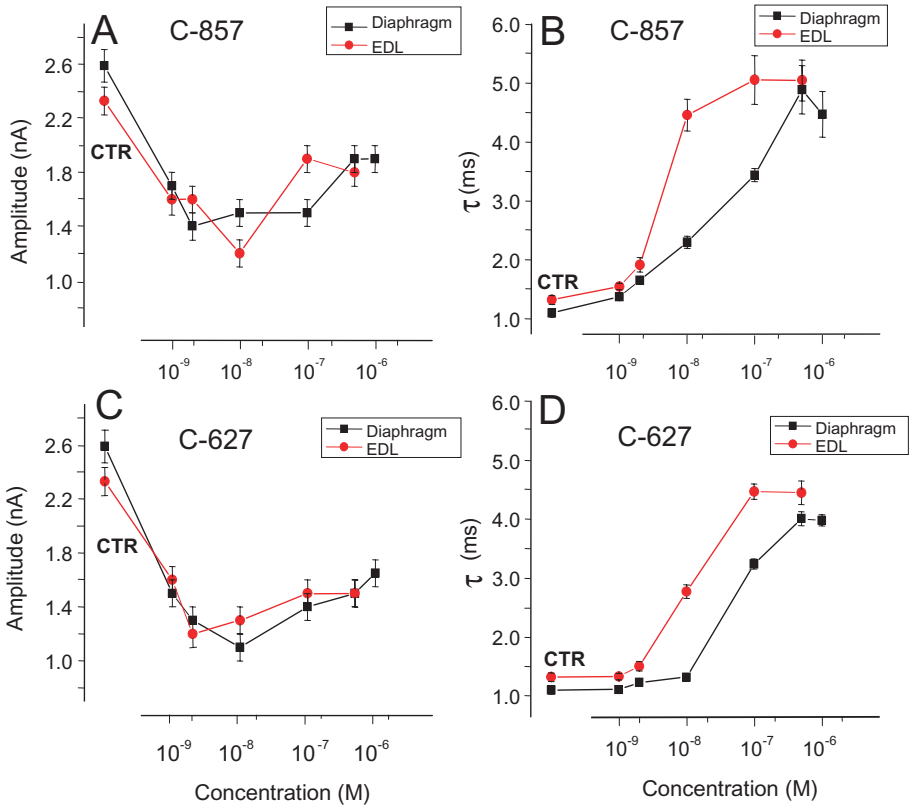


Figure 3

Concentration-dependent effects of C-857 (A, B), C-627 (C, D) on amplitude (in nA) and decay time constant (τ in ms) of MEPC recorded in 10 experiments from diaphragm and extensor digitorum longus (EDL) muscles. CTR, control values.

that BuChE might be more significant for ACh hydrolysis in the diaphragm than in the EDL. The catalytic activity of BuChE and AChE was measured in muscle homogenates and the BuChE and AChE mRNA level in these muscles was quantitatively evaluated. The ratio between the enzymatic activity of the AChE and BuChE was 3.7 in the EDL (0.259 ± 0.005 $U \cdot g^{-1}$ vs. 0.07 ± 0.006 $U \cdot g^{-1}$, 7 muscles) and in the diaphragm it was close to 1 (0.166 ± 0.007 $U \cdot g^{-1}$ vs. 0.155 ± 0.002 $U \cdot g^{-1}$, 7 samples, each from 3 muscles). This corresponds closely to the relative AChE and BuChE mRNA level in the diaphragm and EDL where the ratio was 4.6 in the EDL and 1 in the diaphragm.

C-547 action on diaphragm with inhibited BuChE

If the differences in the efficiency of C-547 could be caused by the higher activity of BuChE in the diaphragm than in the EDL, then inhibition of BuChE before applying these drugs should lower or eliminate these intermuscular differences. The diaphragm muscle samples were therefore pretreated for 30 min with a selective and irreversible inhibitor of BuChE, 50 μM iso-OMPA (Atack *et al.*, 1989), which was then washed out for another 30 min. MEPCs were then recorded before and after C-547 application at concentrations of 5 and 10 nM. These concentrations were normally ineffective in the diaphragm but brought about the maximum prolongation in the EDL (Figure 2A and B) and soleus locomotor muscles (Petrov *et al.*, 2006; 2009). Inhibition of BuChE with iso-OMPA alone or together with 5 nM C-547 did not cause any substantial changes in the amplitude and time course of MEPCs in this muscle (Figure 4). A significant increase in MEPC amplitude (by 29%) and increase in τ (by 43%) only occurred at a higher concentration, 10 nM of C-547. (Figure 4, right hand columns). This higher concentration was, however, ineffective when BuChE was active (Figure 2E and F).

Different AChE sensitivities of the diaphragm and EDL homogenates to C-547

Another reason might be differing sensitivities of the AChE itself in the diaphragm and EDL to C-547. To investigate this possibility, we examined the degree of AChE inhibition by C-547 in EDL and diaphragm homogenates. The contribution of BuChE to the total cholinesterase activity was excluded by adding 50 μM iso-OMPA to the reaction mixture. The half-inhibition concentration of C-547 (IC_{50}) in the EDL obtained by analysis of the dose–effect curve was 3 nM while the IC_{50} for diaphragm AChE was 72 nM (Figure 5).

The G4 form of AChE was less sensitive towards C-547

The differing sensitivities of the AChE from the EDL and diaphragm to C-547 might be due to molecular form

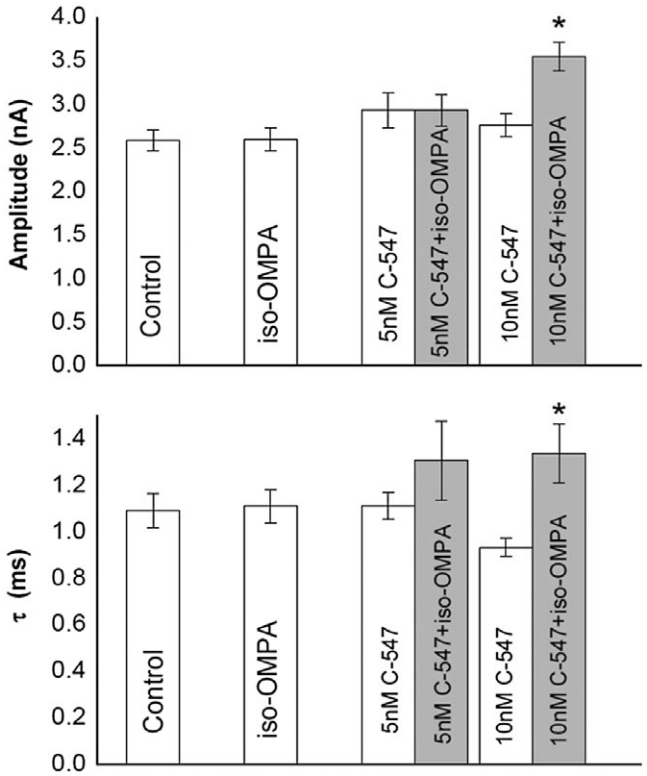


Figure 4

Effects of 5 nM (C, D) and 10 nM C-547 (E, F) on the amplitude (in nA, upper panel) and decay time constant τ (in ms, lower panel) of MEPC recorded in 10 diaphragm fibres, each from a different muscle. For each fibre, 200 MEPCs from diaphragm muscles, either control or pretreated with 50 μM iso-OMPA (a BuChE inhibitor) were averaged, pooled and expressed as mean ± SEM. *P < 0.05, significantly different from values without iso-OMPA.

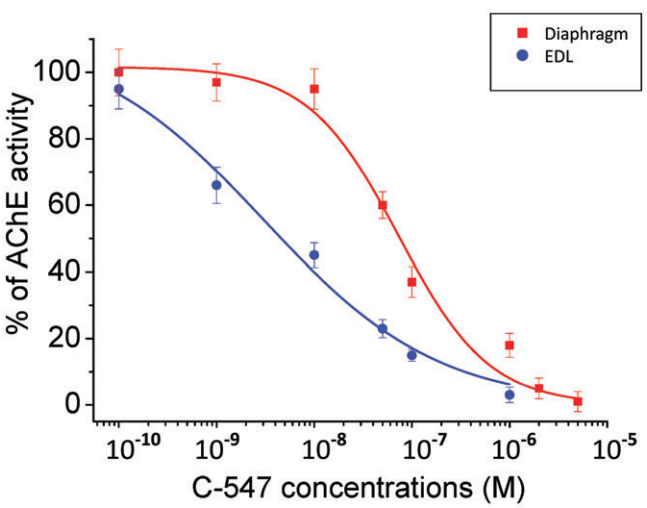


Figure 5

Normalized dose–effect curves for C-547 on AChE activity in EDL and diaphragm homogenates.

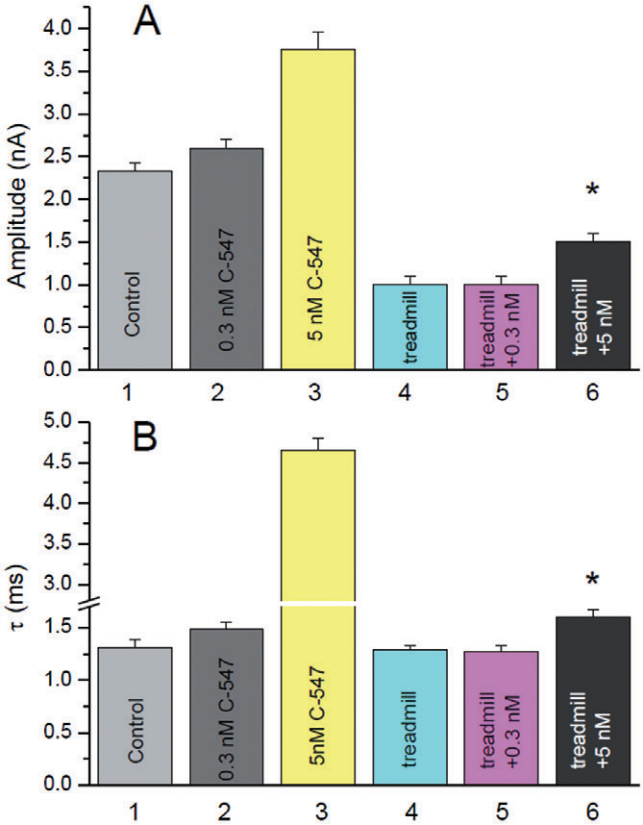


Figure 6

Effects of 0.3 nM and 5 nM C-547 on amplitude (in nA, upper part) and decay time constant (τ in ms, lower part) of MEPC recorded in 6 experiments from EDL, either control or after treadmill exercises. *P < 0.05, significantly different from values without 5 nM C-547.

composition, as endplate-specific AChE has at least two different forms, A12 as the major component and G4 as a minor component (Legay, 2000). The amount of G4, but not A12, can increase during intensive physical activity in fast fibres (Gisiger *et al.*, 1994), which are most prevalent in C-547 -sensitive EDL muscle. Such an activity-induced change of the G4/A12 ratio could influence the sensitivity of EDL muscle to C-547 and thus the effect of this compound on MEPCs was tested in EDL from 'sedentary' control rats and treadmill-trained ones who had several periods of running over 2 days. While the τ of decay remained unchanged after treadmill runs, the MEPC amplitude was significantly decreased. (Figure 6, columns A1 and A4 from the left; P < 0.05). This amplitude decrease might reflect the increased overall anti-cholinesterase activity in the synaptic cleft that can lower the number of non-hydrolysed ACh molecules in the quanta that reach the subsynaptic membrane. Two concentrations of C-547 were then used: 0.3 nM (threshold concentration), which only increased both variables in 'sedentary' rats by several percent, and the most effective concentration of 5 nM, which increases the amplitude and τ of decay to their maximum values (cf. Figure 2A and B, P < 0.05). C-547 at the marginal concentration of 0.3 nM did not change the MEPC amplitude and τ of decay in treadmill-trained EDL (Figure 6, columns 4 and 5).

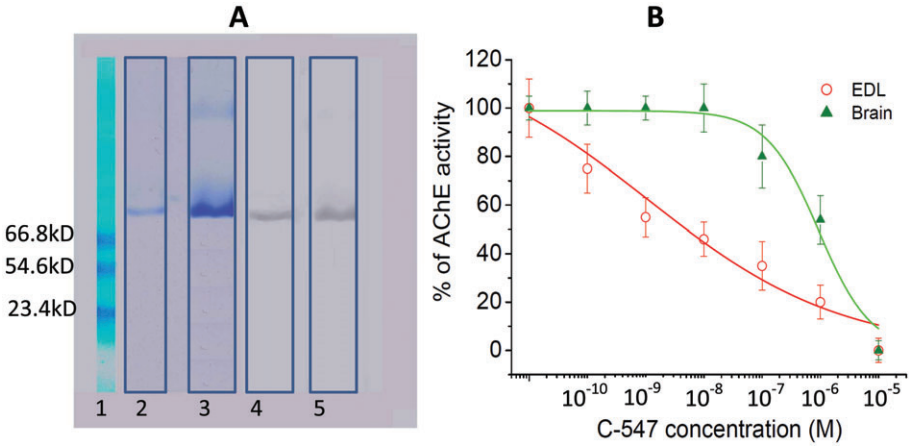


Figure 7

(A) 10% SDS-PAGE of one of the samples used during AChE isolation. Line 1 – ladder of markers, lines 2 and 3 – EDL and brain Coomassie Brilliant Blue labelled protein after purification by ultracentrifugation and gel filtration on Bio Gel A 50 column. Lines 4 and 5 show immunoblotting for EDL and brain, respectively, with E19 antibody to AChE. The main proteins in the samples are AChE monomers of 70–80 kDa. (B) Dose–effect curve for C-547 on purified brain and EDL AChE activity.

The anti-cholinesterase effect of an otherwise fully effective 5 nM dose of C-547 was lower; the MEPC amplitude increase was 61% in sedentary EDL above controls (Figure 6A, compare columns 1 and 3; $P < 0.05$) and only 50% in trained EDLs (Figure 6A, compare columns 4 and 6; $P < 0.05$). The change in C-547 sensitivity in terms of MEPC decay was more obvious and τ increased by only 25% (Figure 6B, columns 4 and 6; $P < 0.05$) in trained EDL compared with 260% in sedentary EDL muscles (Figure 6B, columns 1 and 3; $P < 0.05$). It follows that forced training, which is known to increase the G4 molecular form of AChE, also lowers the anti-cholinesterase effect of C-547.

Biochemical determination of C-547 effectiveness on brain AChE

From the training-induced increase of the G4/A12 ratio and decrease in the sensitivity of EDL to C-547 it could be assumed that C-547 should be less effective on brain AChE, where the G4 molecular form prevails and comprises 80–90% of the total AChE activity (Rotundo, 1988), as already stated. To test this assumption, we measured the IC_{50} for C-547 on purified AChE from the brain and from EDL, after the inhibition of BuChE by 50 μM iso-OMPA. The purity of the isolated brain and EDL proteins was verified by electrophoresis in 10% polyacrylamide gel with subsequent immunoblotting (Figure 7A). The IC_{50} of C-547 was 1 nM for the EDL (Figure 7B), which was close to the previously obtained value for the whole AChE homogenate (3nM, Figure 5). The IC_{50} of C-547 for purified brain AChE was 960 nM (Figure 7), indicating that the sensitivity of the brain AChE is almost three orders of magnitude lower than that of the EDL.

Electrophysiological determination of C-547 efficiency to the brain AChE

It is known that almost 80% of the total AChE activity in homogenized tissue is due to an AChE pool localized intracellularly (Younkin *et al.*, 1982). This intracellular pool of AChE does not participate in the functionally important extracellular pool that hydrolyses the ACh at native synapses. We estimated the sensitivity of the latter pool of AChE to C-547 in electrophysiological experiments on hippocampus slices.

Activation of muscarinic acetylcholine receptors *in vitro* modulates immature hippocampus network activity and induces network oscillations that can be recorded by fine tungsten electrodes in the hippocampus, specifically in the CA3 subfield of the pyramidal cell layer. Using hippocampal slices from 16–25 days old animals, we compared the non-specific anti-AChE agent, neostigmine and the tissue-specific C-547 compounds, in terms of their ability to induce synchronous network activity via the elevation of ACh level as a result of cholinesterase inhibition (Figure 8) (Fellous and Sejnowski, 2000).

No oscillatory bursts of spikes were observed in the absence or presence of 2 μM neostigmine. Therefore, slices were superfused with 70 μM ACh before neostigmine application. This concentration was selected in preliminary experiments as best for disclosing the anti-AChE action of the drugs. 70 μM ACh did not evoke any synchronized activity by itself; however, neostigmine induced regular network discharges in the CA3 hippocampal region in the presence of ACh with a frequency of 0.9 ± 0.2 Hz, amplitude of 0.94 ± 0.20 mV and half-width of 19 ± 3 ms ($n = 4$, Figure 8A3 and left inset), which was inhibited by 10 μM of the muscarinic acetylcholine receptor antagonist atropine (Figure 8A4). In contrast to neostigmine, C-547 in concentrations that were effective on the EDL (5–100 nM), evoked no synchronized network activity when it was applied simultaneously with ACh (Figure 8B2). The lack of effect of C-547 at these concentrations could be caused by the activity of C-547-insensitive BuChE. To test this possibility, C-547 was applied together with 50 μM iso-OMPA. No network discharges were observed under these conditions either (data not shown).

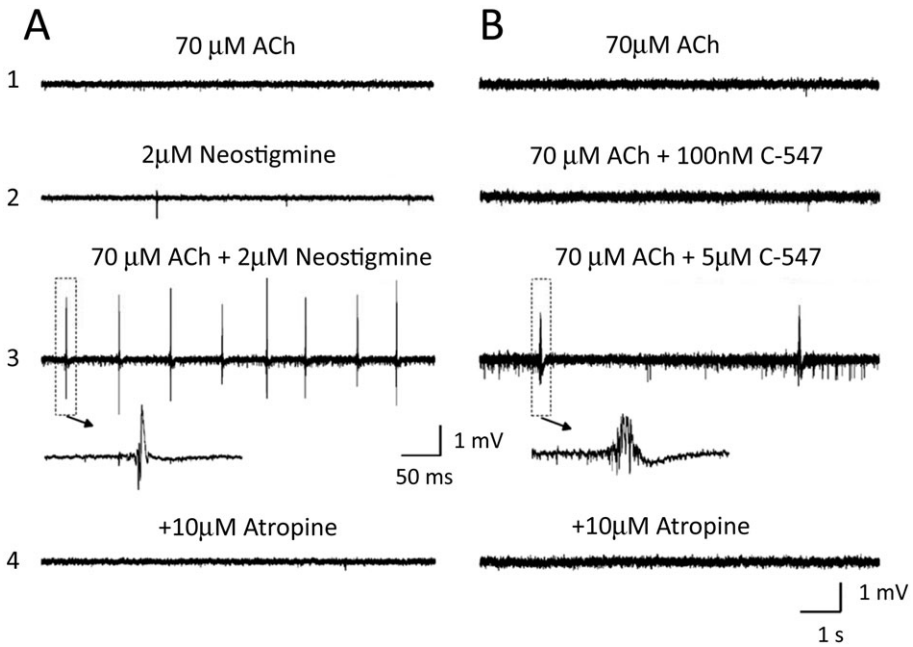


Figure 8

Field recordings of slow rhythmic activity in 70 mM ACh and neostigmine (A) and in 70 mM ACh and C-547 (B) in hippocampal cell body layer of CA3. Calibration for records 1–4 is bottom right, for extended bursts in the middle. Bottom records show disappearance of activity when atropine was added to the superfusing solution already containing 70 mM ACh and neostigmine (A4) or C-547 (B4).

C-547 only exhibited its activity at relatively high concentrations (1–10 µM). After application of 5 µM C-547, regular network activity appeared with a mean amplitude of 0.67 ± 0.22 mV, half-width of 24 ± 3 ms and frequency of 0.3 ± 0.1 Hz (n = 4, Figure 8B3 and right inset), which was similar to that observed after 2 µM neostigmine; it was also atropine-sensitive (Figure 8B4) but firing occurred with lower frequency

Relative expression of AChE-R variant in EDL, diaphragm and brain

Besides the anchored forms of AChE (A12, G4), the soluble monomer AChE-R can be present in the synaptic cleft. This monomer is a 'read-through' transcript of the 4th exon of the AChE gene. It has no sequence for the formation of the complex with anchor subunits, but has similar catalytic properties to anchored forms of AChE and may contribute to the rate of ACh hydrolysis in the synaptic cleft. In this series of experiments, we investigated the possible correlation between the expression levels of AChE-R and sensitivity to C-547.

Analysis of AChE-R mRNA revealed no significant differences in the levels of the invariable exon 2 (A) and read-through transcript (B) of the EDL, diaphragm and brain samples (Figure 9).

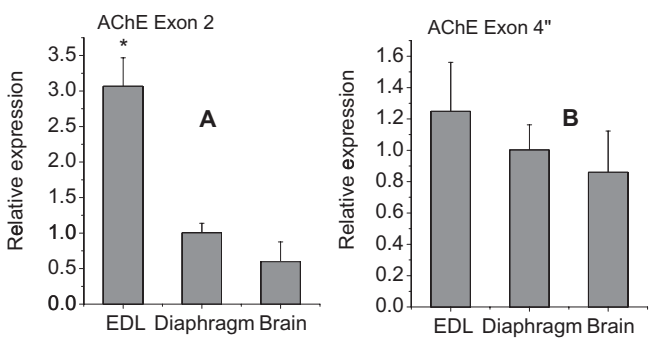


Figure 9

Relative expression of invariable exon 2 (A) and read-through transcript (B) of 4th exon of AChE in the EDL and brain compared with diaphragm. Level in the diaphragm was taken as 1. *P < 0.05, significantly different from expression in diaphragm or brain.

Discussion

The compound C-547, a member of the ADEMS set, increased the amplitude and decay time of MEPC in a manner characteristic of AChE inhibition. However, it was effective in the diaphragm and in external intercostal muscles at concentrations that are greater by one or two orders of magnitude than on the EDL and soleus locomotor muscles (Petrov *et al.*, 2006; 2009). In this work, two other ADEMS compounds, C-627 and C-857, were compared with C-547. Like C-547, these analogues also exhibited a greater potency on the EDL, in particular, the compound C-857, which has only one alkylammonium radical in position 1 of the uracil ring. This position is evidently more important than position 3 for observed sensitivity differences and points to fine variations in the cholinesterase binding sites between diaphragm and

EDL muscles. Such variations were not detected with the other AChE inhibitors neostigmine, paraoxon and armin. Hence, the difference between respiratory and locomotor muscles is characteristic of ADEMS as AChE molecular sensors rather than the result of a differing accessibility of diaphragm and leg muscles to anti-cholinesterase agents, in general. Here we showed that there are two possible explanations for our findings a different ratio of ADEMS-sensitive AChE to ADEMS-insensitive BuChE and/or different sensitivities of the AChE subtypes.

The family of mammalian cholinesterases includes two closely related enzymes, capable of hydrolysing ACh and, consequently, of controlling its concentration in the synaptic cleft. These are AChE (EC 3.1.1.7) and BuChE (3.1.1.8). Irrespective of the fact that 65% of the amino acid sequences of these enzymes are homologous and the catalytic centres are virtually identical, they differ substantially in the efficiency of substrate hydrolysis and kinetics of their interaction with a number of inhibitors (Chatonnet and Lockridge, 1989). It is known that BuChE hydrolyses ACh at high concentrations and is not inhibited by an excess of substrate, while AChE functions better at a relatively low substrate concentration and is inhibited by an excess of substrate (Zdrazilova *et al.*, 2006). Hence, it is generally accepted that the rapid hydrolysis of ACh released from nerve motor endings is mostly performed by AChE while the synaptic function of BuChE is not quite clear. BuChE could substitute for the AChE when the latter is insufficient (Minic *et al.*, 2003; Girard *et al.*, 2007). There is a decrease in AChE activity and simultaneously an increase in BuChE activity during Alzheimer's and Parkinson's diseases and during cerebral trauma (Giacobini, 2004). Importantly, addition of horse serum BuChE or recombinant human BuChE into the systemic circulation was able to protect animals from lethal doses of AChE inhibitors (Lenz *et al.*, 2007; Masson and Lockridge, 2010).

As C-547 is a selective inhibitor of AChE (Anikienko *et al.*, 2008) the activity of BuChE in the diaphragm could compensate for the loss of AChE activity. This possibility was supported by the fact that the level of mRNA of BuChE and the activity of the enzyme is several times greater in the diaphragm than in the EDL. Undoubtedly, C-547-resistant BuChE in the diaphragm can significantly counteract the prolongation of MEPCs. However, the elimination of BuChE before the application of C-547 increased the sensitivity of the diaphragm to C-547 but did not fully remove the differences between the EDL and diaphragm. In other words, without iso-OMPA, a proportion of the diaphragm AChE is already inhibited by 10 nM C-547. Because ACh is still hydrolysed by BuChE, the MEPCs are not prolonged. After the elimination of BuChE with iso-OMPA, 10 nM C-547 inhibits the diaphragm AChE sufficiently to increase the amplitude and time course of the MEPCs. Because this was not observed with the lower, 5 nM concentration of C-547 known to be fully effective on EDL (Figure 2E and F), BuChE activity can be viewed as important but not the only reason for the lower diaphragm sensitivity to ADEMS. Therefore, there must be another mechanism underlying the lower sensitivity of the diaphragm to the ADEMS and our assays of AChE in EDL and diaphragm homogenates showed that diaphragm AChE was actually more than 20 times less sensitive to C-547 than that from the EDL.

What could be the basis of this difference in AChE sensitivity? In vertebrates, AChE is coded by a single gene (Hasin *et al.*, 2004; Krejci *et al.*, 2006; Massoulie *et al.*, 2008), but there is a heterogeneity of the molecular forms of AChE as a result of alternative splicing, the attachment of various non-catalytic subunits and post-translational modifications. In mammals, several alternative splicing variants of the AChE gene are known, as well as a single read-through transcript (Legay, 2000), Variants of the alternative splicing of the 5th or 6th exon usually designated 'E' and 'S' generate catalytic subunits, which contain the same catalytic domain associated with distinct C-terminal peptides. So, the first variant 'E' (the so-called 'erythrocytic' variant) codes the 32 amino acids at the C-terminus that is responsible for the attachment of the glycophosphatidylinositol anchor. The 'E' variant was found mainly at the surface of hematopoietic cells but the physiological function of the enzyme in these cells remains unclear. The 'S' variant (AChE-S) is expressed in the CNS as well as in skeletal muscles. This variant (the so-called 'synaptic' or 'tailed' variant) codes 40 amino acids in the C-terminus, which form a specific domain with the cysteine residue located three amino acids from the end of the protein. This cysteine residue enables disulfide bonding with other AChE subunits, giving rise to amphipathic homodimers and homotetramers that can interact with anchor subunits that are specific for this variant.

In addition, several reports describe alternative promoter usage and splicing at the 5′-end of the gene (Meshorer *et al.*, 2004). However, these sequences located before the translation initiation ATG codon are absent in the AChE protein, and probably play a regulatory role.

In addition, in some species, including mice, rats and humans, a read-through transcript (AChE-R) of the 4th exon has been described. The C-terminus of AChE-R differs from those of other AChE variants and cannot tetramerize via disulfide bonding with other subunits, and so only produces a soluble monomeric molecule. The other 95% of the coding sequences of AChE are invariant, as is the catalytic domain of all AChE isoforms.

The AChE-R variant was found in hematopoietic cells of mice and rats but only expressed as 1% of the total AChE in electrically excitable cells under normal conditions. The expression of this variant rapidly increased (during 30 min) in the CNS during stress (Perrier *et al.*, 2002) and in muscles during myasthenia gravis (Brenner *et al.*, 2003). After exposure to anti-AChE agents, the AChE-R level slightly increases but remains minor (around 1%) (Perrier *et al.*, 2005). Direct injection of human BuChE into the circulation provides protection against organophosphate poisoning (Evron *et al.*, 2007), but the possibility of the involvement of soluble monomers of AChE-R in the rapid hydrolysis of ACh in the synaptic cleft remains an open question. For example, in mutant animals without AChE-S in the synapses, no changes in the amplitude and duration of synaptic potentials after AChE inhibitors were observed. This suggests that in the absence of AChE-S in the synaptic cleft, inhibitors of AChE did not cause a further increase in ACh concentration and, hence, the contribution of AChE-R to the hydrolysis of ACh, in these mutants at least, is not significant. Our PCR analysis of the AChE-R mRNA expression of this minor AChE variant showed no significant differences between all of the investi-

gated organs. Because the differences in the sensitivity of AChE from the various organs to C-547 were observed in both electrophysiological and biochemical experiments, we can exclude the putative enhancement of AChE-R synthesis in response to the inhibition of AChE-S as a hypothesis to explain the differences in sensitivity to C-547. Thus, our analysis revealed no correlation between the efficiency of AChE inhibition in various organs by C-547 and the level of mRNA splicing (AChE-S vs. AChE-R).

Homotetramers of AChE-S can covalently bind with one of the anchor subunits – Col Q (collagen Q) or PRiMA (proline-rich membrane anchor) (Massoulie, 2002). ColQ forms a triple helical structure in a proline-rich domain in their C-terminus. As each ColQ can attach one AChE-S tetramer, hetero-oligomeric complexes may contain four, eight or twelve AChE subunits, which are designated the A4, A8 and A12 asymmetric forms, respectively (Bon *et al*. 2003). In muscles, the dominant one is A12, which consists of three tetramers anchored at the basal lamina via the collagen-like tail Col Q. The latter is a minority tetramer bound to the cell membrane by 20 kDA – PRiMA and its content varies in different types of muscles. On the other hand, G4 is highly abundant in the brain where it forms 80–90% of the total cholinesterase activity (Rotundo, 1988; Inestrosa *et al*., 1994). PRiMA induces the formation of the complex with AChE-S homotetramers (G4) and attaches them to the cell membrane via a transmembrane domain (Perrier *et al*., 2002). The PRiMA anchor is mainly synthesized by nervous tissue (Perrier *et al*., 2002; 2003) but Col Q by muscles (Feng *et al*., 1999). In muscle cells, the expression of PRiMA mRNA, as well as the level of PRiMA AChE was suppressed by myogenesis and innervation (Xie *et al*., 2008). Therefore, in neuromuscular junctions, the majority of the AChE is anchored to the basal lamina by Col Q with a relatively low level of PRiMA AChE that is most likely secreted by the nerve (Jevsek *et al*., 2004; Leung *et al*., 2009). In brain synapses, the enzyme is mainly attached to the plasma membrane through PRiMA (Grassi *et al*., 1982); moreover, this anchor is required for the intracellular processing of AChE in neurons (Dobbertin *et al*., 2009). However, the specific inhibitors for the catalytic subunits formed by Col Q and PRiMA-anchored molecular forms of AChE are not known. In this respect, it is interesting that treadmill exercise, which increases the amount of PRiMA AChE (G4), but not Col Q AChE (A12) in fast EDL, can dramatically decrease the sensitivity of the EDL to C-547. This suggests that AChE anchored by PRiMA is less sensitive to C-547 than the AChE anchored by Col Q. Therefore, it is not surprising that brain PRiMA-anchored AChE is three orders of magnitude less sensitive to C-547 (as we have demonstrated here in biochemical and electrophysiological experiments) than it would be if it were anchored by Col Q. Most likely the G4 form is less sensitive towards the inhibitory action of this compound. However, the purification and direct measurement of the sensitivity of isolated G4 to C-547 is a difficult task because of the relatively low level of activity of this subtype in the total muscular AChE. Experiments in this regard are underway.

What then is known about the differences between PRiMA and ColQ AChEs? ColQ and PRiMA appear to form similar but not the same quaternary complexes with AChE-S subunits, which differ in the number of prolines in the domains that form this complex (8 in ColQ, 14 in PRiMA). In addition, the number and positions of the cysteines are very different in ColQ (2 adjacent cysteines, located 4 and 5 residues upstream of the first proline) and in PRiMA (4 cysteines located 2, 4, 8 and 15 residues upstream of the first proline, plus a cysteine located 13 residues downstream of the last proline) (Massoulie, 2002). Therefore, the organization of the complex of AChE with one of the anchor subunits (PRiMA and ColQ) may contain some structural differences, but it is not yet known how these may influence the interaction with AChE inhibitors (in particular, with C-547).

Differences between the catalytic subunits of PRiMA and ColQ AChEs were described at the level of the post-translational modification of AChE. So, human, mouse and rat AChE carries three N-glycosylation sites, all of which were utilized in the recombinant version of the enzyme. Using lectin-agarose conjugates, it has been demonstrated that AChE anchored by Col Q binds to DBA and VVA-B lectins (specific for N-acetylgalactosamine), while AChE anchored by PRiMA does not (Scott *et al*., 1988). This means that the terminal residues in the N-glycans of PRiMA and ColQ AChE isoforms are different. These are N-acetylgalactosamine in ColQ AChE and another N-glycan in AChE anchored by PRiMA. Although differences in glycosylation have been shown to affect the efficiency of some AChE inhibitors (edrophonium, rivastigmine) or 2G8 antibody (Liao *et al*., 1991), the involvement of glycosylation in the differing sensitivities of PRiMA and ColQ AChEs to ADEMS requires verification.

To our knowledge, this report is the first evidence of the possibility of organ- and tissue-specific AChE inhibition. It also raises the issue of the molecular differences between the PRiMA and ColQ AChEs and, consequently, of the possibility of the targeted synthesis of new anti-cholinesterase agents, specific to the brain, say, for the treatment of Alzheimer's disease. At the same time, muscle-specific ADEMS could be tested for the ability to treat syndromes caused by either a decreased density of cholinoceptors or amount of transmitter released, such as myasthenia gravis, a number of congenital myasthenic syndromes (Engel *et al*., 2010) or Sjogren's syndrome with malfunctioning salivation (Dawson *et al*., 2005). Also patients with brain injuries (Giacobini, 2004) or in a post-stroke condition, whose AChE levels are reduced but BuChE levels elevated (Ben Assayag *et al*., 2010) may be affected by the various ADEMS in different ways. It would also be important to test whether the carriers of common debilitating variants of BuChE, such as the Kalow variants in the BuChE gene, having a 30% lower catalytic activity that might be a potential contributing factor to Alzheimer's disease) (Podoly *et al*., 2009) could be less susceptible to the effects of ADEMS.

Future experimental studies should be focused: (i) on the possible modulation of ADEMS binding to Col Q- vs. PRiMA-AChEs; (ii) on the differences in AChE N-glycosylation; and (iii) on uncovering other reasons for the differing sensitivities of AChEs to these compounds.

Acknowledgements

The authors thank Dr Rustem Khazipov for suggesting experiments on hippocampus slices and reading the manuscript.

This study was supported by RFBR Grant 09-04-12047, Scientific school 64631.2010.7 'Fundamental Sciences in Medicine', Regional Center of Collective Use, Kazan (Volga Region) and Federal University, Kazan, Russia. R. A. A. was supported in part by NATO reintegration Grant NR.RIG.983007. The contribution of F.V. was covered by IAA500110905, GAČR 202/09/0806 and AV0Z 0110509.

Conflict of interest

The authors declare no conflicts of interest.

References

Anikienko KA, Bychikhin EA, Reznik VS, Akamsin D, Galyametdinova IV (2008). Compounds with the dioxopyrimidine cycle inhibit cholinesterases from different groups of animals. Chem Biol Interact 175: 286–292.

Atack JR, Yu QS, Soncrant TT, Brossi A, Rapoport SI (1989). Comparative inhibitory effects of various physostigmine analogs against acetyl- and butyrylcholinesterases. J Pharmacol Exp Ther 249: 194–202.

Ben Assayag E, Shenhar-Tsarfaty S, Ofek K, Soreq L, Bova I, Shopin L *et al.* (2010). Serum cholinesterase activities distinguish between stroke patients and controls and predict 12-month mortality. Mol Med 16: 278–286.

Bon S, Ayon A, Leroy J, Massoulie J (2003). Trimerization domain of the collagen tail of acetylcholinesterase. Neurochem Res 28: 523–535.

Brenner T, Hamra-Amitay Y, Evron T, Boneva N, Seidman S, Soreq H (2003). The role of readthrough acetylcholinesterase in the pathophysiology of myasthenia gravis. FASEB J 17: 214–222.

Caldwell JE (2009). Clinical limitations of acetylcholinesterase antagonists. J Crit Care 24: 21–28.

Chatonnet A, Lockridge O (1989). Comparison of butyrylcholinesterase and acetylcholinesterase. Biochem J 260: 625–634.

Dawson LJ, Caulfield VL, Stanbury JB, Field AE, Christmas SE, Smith PM (2005). Hydroxychloroquine therapy in patients with primary Sjogren's syndrome may improve salivary gland hypofunction by inhibition of glandular cholinesterase. Rheumatology (Oxford) 44: 449–455.

Dobbertin A, Hrabovska A, Dembele K, Camp S, Taylor P, Krejci E *et al.* (2009). Targeting of acetylcholinesterase in neurons in vivo: a dual processing function for the proline-rich membrane anchor subunit and the attachment domain on the catalytic subunit. J Neurosci 29: 4519–4530.

Doležal V, Vyskočil F, Tuček S (1983). Decrease of the spontaneous non-quantal release of acetylcholine from the phrenic nerve in botulinum-poisoned rat diaphragm. Pflugers Arch 397: 319–322.

Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7: 88–95.

Engel AG, Shen XM, Selcen D, Sine SM (2010). What have we learned from the congenital myasthenic syndromes. J Mol Neurosci 40: 143–153.

Evron T, Geyer BC, Cherni I, Muralidharan M, Kilbourne J, Fletcher SP *et al.* (2007). Plant-derived human acetylcholinesterase-R provides protection from lethal organophosphate poisoning and its chronic aftermath. FASEB J 21: 2961–2969.

Fellous JM, Sejnowski TJ (2000). Cholinergic induction of oscillations in the hippocampal slice in the slow (0.5–2 Hz), theta (5–12 Hz), and gamma (35–70 Hz) bands. Hippocampus 10: 187–197.

Feng G, Krejci E, Molgo J, Cunningham JM, Massoulie J, Sanes JR (1999). Genetic analysis of collagen Q: roles in acetylcholinesterase and butyrylcholinesterase assembly and in synaptic structure and function. J Cell Biol 144: 1349–1360.

Giacobini E (2004). Cholinesterase inhibitors: new roles and therapeutic alternatives. Pharmacol Res 50: 433–440.

Giniatullin RA, Khazipov RN, Oranska TI, Nikolsky EE, Voronin VA, Vyskočil F (1993). The effect of non-quantal acetylcholine release on quantal miniature currents at mouse diaphragm. J Physiol 466: 105–114.

Giniatullin RA, Talantova MV, Vyskočil F (2001). The role of desensitisation in decay time of miniature endplate currents in frogs Rana ridibunda and Rana temporaria. Neurosci Res 39: 287–292.

Girard E, Bernard V, Minic J, Chatonnet A, Krejci E, Molgo J (2007). Butyrylcholinesterase and the control of synaptic responses in acetylcholinesterase knockout mice. Life Sci 80: 2380–2385.

Gisiger V, Belisle M, Gardiner PF (1994). Acetylcholinesterase adaptation to voluntary wheel running is proportional to the volume of activity in fast, but not slow, rat hindlimb muscles. Eur J Neurosci 6: 673–680.

Grassi J, Vigny M, Massoulie J (1982). Molecular forms of acetylcholinesterase in bovine caudate nucleus and superior cervical ganglion: solubility properties and hydrophobic character. J Neurochem 38: 457–469.

Hasin Y, Avidan N, Bercovich D, Korczyn A, Silman I, Beckmann JS *et al.* (2004). A paradigm for single nucleotide polymorphism analysis: the case of the acetylcholinesterase gene. Hum Mutat 24: 408–416.

Inestrosa NC, Perez CA, Simpfendorfer RW (1994). Sensitivity of acetylcholinesterase molecular forms to inhibition by high MgCl2 concentration. Biochim Biophys Acta 1208: 286–293.

Jevsek M, Mars T, Mis K, Grubic Z (2004). Origin of acetylcholinesterase in the neuromuscular junction formed in the in vitro innervated human muscle. Eur J Neurosci 20: 2865–2871.

Koelle GB, Davis R, Diliberto EJ Jr, Koelle WA (1974). Selective, near-total, irreversible inactivation of peripheral pseudocholinesterase and acetylcholinesterase in cats in vivo. Biochem Pharmacol 23: 175–188.

Kovyazina IV, Nikolsky EE, Giniatullin RA, Adamek S, Vyskočil F (2003). Dependence of miniature endplate current on kinetic parameters of acetylcholine receptors activation: a model study. Neurochem Res 28: 443–448.

Kovyazina IV, Petrov KA, Zobov VV, Bukharaeva EA, Nikolsky EE (2004). Effect of tetraalkylammonium derivatives of 6-methyluracil on the endplate potentials of muscles of different functional types. Dokl Biol Sci 399: 458–460.

Krejci E, Martinez-Pena y Valenzuela I, Ameziane R, Akaaboune M (2006). Acetylcholinesterase dynamics at the neuromuscular junction of live animals. J Biol Chem 281: 10347–10354.

Kuba K, Tomita T (1971). Effect of prostigmine on the time course of the end-plate potential in the rat diaphragm. J Physiol 213: 533–544.

Land BR, Harris WV, Salpeter EE, Salpeter MM (1984). Diffusion and binding constants for acetylcholine derived from the falling phase of miniature endplate currents. Proc Natl Acad Sci USA 81: 1594–1598.

Laskowski MB, Dettbarn WD (1979). An electrophysiological analysis of the effects of paraoxon at the neuromuscular junction. J Pharmacol Exp Ther 210: 269–274.

Legay C (2000). Why so many forms of acetylcholinesterase? Microsc Res Tech 49: 56–72.

Lenz DE, Yeung D, Smith JR, Sweeney RE, Lumley LA, Cerasoli DM (2007). Stoichiometric and catalytic scavengers as protection against nerve agent toxicity: a mini review. Toxicology 233: 31–39.

Leung KW, Xie HQ, Chen VP, Mok MK, Chu GK, Choi RC *et al.* (2009). Restricted localization of proline-rich membrane anchor (PRiMA) of globular form acetylcholinesterase at the neuromuscular junctions – contribution and expression from motor neurons. FEBS J 276: 3031–3042.

Liao J, Heider H, Sun MC, Stieger S, Brodbeck U (1991). The monoclonal antibody 2G8 is carbohydrate-specific and distinguishes between different forms of vertebrate cholinesterases. Eur J Biochem 198: 59–65.

Lupa MT, Tabti N, Thesleff S, Vyskocil F, Yu SP (1986). The nature and origin of calcium-insensitive miniature end-plate potentials at rodent neuromuscular junctions. J Physiol 381: 607–618.

Masson P, Lockridge O (2010). Butyrylcholinesterase for protection from organophosphorus poisons: catalytic complexities and hysteretic behavior. Arch Biochem Biophys 494: 107–120.

Massoulie J (2002). The origin of the molecular diversity and functional anchoring of cholinesterases. Neurosignals 11: 130–143.

Massoulie J, Perrier N, Noureddine H, Liang D, Bon S (2008). Old and new questions about cholinesterases. Chem Biol Interact 175: 30–44.

Meshorer E, Toiber D, Zurel D, Sahly I, Dori A, Cagnano E *et al.* (2004). Combinatorial complexity of 5′ alternative acetylcholinesterase transcripts and protein products. J Biol Chem 279: 29740–29751.

Minic J, Chatonnet A, Krejci E, Molgo J (2003). Butyrylcholinesterase and acetylcholinesterase activity and quantal transmitter release at normal and acetylcholinesterase knockout mouse neuromuscular junctions. Br J Pharmacol 138: 177–187.

Nikolsky EE, Oranska TI, Vyskocil F (1992). Non-quantal acetylcholine release after cholinesterase inhibition in vivo. Physiol Res 41: 333–334.

Perrier AL, Massoulie J, Krejci E (2002). PRiMA: the membrane anchor of acetylcholinesterase in the brain. Neuron 33: 275–285.

Perrier NA, Kherif S, Perrier AL, Dumas S, Mallet J, Massoulie J (2003). Expression of PRiMA in the mouse brain: membrane anchoring and accumulation of 'tailed' acetylcholinesterase. Eur J Neurosci 18: 1837–1847.

Perrier NA, Salani M, Falasca C, Bon S, Augusti-Tocco G, Massoulie J (2005). The readthrough variant of acetylcholinesterase remains very minor after heat shock, organophosphate inhibition and stress, in cell culture and in vivo. J Neurochem 94: 629–638.

Petrov K, Kovyazina I, Zobov V, Bukharaeva E, Nikolsky EE, Vyskocil F (2009). 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. Physiol Res 58: 149–153.

Petrov KA, Kovyazina LV, Zobov VV, Bukharaeva EA, Nikolsky EE, Vyskocil F (2006). Different sensitivity of miniature endplate currents of the rat extensor digitorum longus, soleus and diaphragm muscles to a novel acetylcholinesterase inhibitor C-547. Physiol Res 55: 585–589.

Podoly E, Shalev DE, Shenhar-Tsarfaty S, Bennett ER, Ben Assayag E, Wilgus H *et al.* (2009). The butyrylcholinesterase K variant confers structurally derived risks for Alzheimer pathology. J Biol Chem 284: 17170–17179.

Rotundo RL (1988). Biogenesis of acetylcholinesterase molecular forms in muscle. Evidence for a rapidly turning over, catalytically inactive precursor pool. J Biol Chem 263: 19398–19406.

Scott LJ, Bacou F, Sanes JR (1988). A synapse-specific carbohydrate at the neuromuscular junction: association with both acetylcholinesterase and a glycolipid. J Neurosci 8: 932–944.

Shabunova I, Vyskocil F (1982). Postdenervation changes of intracellular potassium and sodium measured by ion selective microelectrodes in rat soleus and extensor digitorum longus muscle fibres. Pflugers Arch 394: 161–164.

Urazaev AK, Magsumov ST, Poletayev GI, Nikolsky EE, Vyskocil F (1995). Muscle NMDA receptors regulate the resting membrane potential through NO-synthase. Physiol Res 44: 205–208.

Vyskocil F, Nikolsky E, Edwards C (1983). An analysis of the mechanisms underlying the non-quantal release of acetylcholine at the mouse neuromuscular junction. Neuroscience 9: 429–435.

Xie HQ, Leung KW, Chen VP, Lau FT, Liu LS, Choi RC *et al.* (2008). The promoter activity of proline-rich membrane anchor (PRiMA) of globular form acetylcholinesterase in muscle: suppressive roles of myogenesis and innervating nerve. Chem Biol Interact 175: 79–82.

Younkin SG, Rosenstein C, Collins PL, Rosenberry TL (1982). Cellular localization of the molecular forms of acetylcholinesterase in rat diaphragm. J Biol Chem 257: 13630–13637.

Zdrazilova P, Stepankova S, Vranova M, Komers K, Komersova A, Cegan A (2006). Kinetics of total enzymatic hydrolysis of acetylcholine and acetylthiocholine. Z Naturforsch [C] 61: 289–294.

Zobov VV, Petrov KA, Asliamova AA, Beresinskii LA, Akamsin VD, Galiametdinova IV *et al.* (2005). Selective blockade of locomotor muscles by uracil-containing tetraalkylammonium acetylcholinesterase inhibitors. Dokl Biol Sci 401: 77–80.