Mechanisms of Cardiac Muscle Insensitivity to a Novel Acetylcholinesterase Inhibitor C-547

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Abstract: We compared the effects of the novel acetylcholinesterase (AChE) inhibitor C-547 on action potential configuration and sinus rhythm in the isolated right atrium preparation of rat with those of armin and neostigmine. Both armin (10^{-7}, 10^{-6}, and 10^{-5} M) and neostigmine (10^{-7}, 10^{-6}, and 5×10^{-6} M) produced a marked decrease in action potential duration and slowing of sinus rate. These effects were abolished by atropine and are attributable to the accumulation of acetylcholine in the myocardium. The novel selective AChE inhibitor C-547 (10^{-9} to 10^{-7} M), an alkylammonium derivative of 6-methyluracil, had no such effects. The inhibition constant of C-547 on cardiac AChE is 40-fold higher than that on *extensor digitorum longus* muscle AChE. These results suggest that C-547 might be employed to treat diseases such as myasthenia gravis or Alzheimer disease, without having unwanted effects on the heart.

Key Words: acetylcholinesterase, action potential, atrium, heart, inhibitor

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INTRODUCTION

Because acetylcholinesterase (AChE) is critically involved in cholinergic synaptic transmission, modulation of its activity by different compounds is of great interest. Although anticholinesterases form one of the largest groups of bioactive compounds used to treat cholinergic failure (eg, myasthenia gravis, Alzheimer disease), these compounds exert effects on the heart, which often are undesirable. Therefore, the application of AChE inhibitors is rather limited, and the search for novel and safer anticholinesterases remains crucial. Recently, a new class of highly selective mammalian AChE inhibitors (alkylammonium derivatives of 6-methyluracil) was synthesized. The ratio of inhibition constants of AChE and butyrylcholinesterase (BuChE) by the most active alkylammonium derivatives of 6-methyluracil can reach 5 orders of magnitude.[1]

During treadmill experiments on dogs and rats, these compounds displayed peculiar features. One of the important properties of these compounds is the great difference between doses required to paralyze respiratory muscles and doses that block limb muscles.[2] Moreover, the threshold concentration of C-547 (one of the most effective derivatives of 6-methyluracil) required for the modulation of amplitude and the duration of synaptic responses typical for full AChE inhibition is 100-fold higher in respiratory muscles than in locomotory muscles.[3] Thus, synapses in the diaphragm are much more resistant to this compound than those in limb muscles.

Investigation of the action of C-547 in heart muscle is of special interest because the parasympathetic nervous system is central to the control of cardiac rate and rhythm and importantly influences contraction as well. For example, parasympathetic input may protect the myocardium during ischemia and reduce the probability of ventricular fibrillation after coronary occlusion.[4] Because AChE inhibitors amplify parasympathetic effects, they are potential cardioprotective agents. Because tissue-specific AChE inhibitors are not currently available, we tested the specificity of C-547, and compared it with the 2 AChE inhibitors: armin (organophosphorus inhibitor) and neostigmine (carbamate inhibitor). We performed experiments on electrical activity of rat atrial myocardium because the atrium is highly sensitive to acetylcholine (ACh). We tested the hypothesis that C-547 has no effect on the myocardium, in which case its potential use in treatment of diseases such as myasthenia gravis may provide a major benefit.

METHODS

All animal experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996), and the experimental protocol was approved by the Animal Care and Use Committee of Kazan State Medical University and Moscow State University.

Transmembrane Potential Recordings of Spontaneous Action Potentials in Isolated Sinoatrial Node

Male Wistar rats were decapitated, the chest was opened, and the heart was rapidly excised and immersed in an

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oxygenated physiological solution containing (in mM) NaCl 130.0, KCl 5.6, NaH_2PO_4 0.6, $MgCl_2$ 1.1, $CaCl_2$ 1.8, $NaHCO_3$ 20.0, and glucose 11.0 bubbled with carbogen (95% O_2 and 5% CO_2) with pH 7.4 ± 0.2. The right atrial preparation including the auricle, the crista terminalis and the intercaval region and sinoatrial node was isolated and pinned to the bottom of an experimental chamber supplied with a physiological solution at 10 mL/min (37.5°C). After 2 hours of equilibration, transmembrane potentials were recorded with glass microelectrodes (20–30 MΩ) filled with 3 M KCl connected to a high-input impedance amplifier. The signal was digitized and analyzed using specific software (L-card, Moscow, Russia; Synaptosoft). Spontaneously occurring action potentials (APs) were recorded from the endocardial surface of the auricle. Stable impalements were maintained during the entire period of drug action. Changes in the cycle length (CL) and the AP duration to 50% of repolarization (APD50) and 90% of repolarization (APD90) were determined.

AChE Activity Measurement

The rat heart and *Musculus extensor digitorum longus* (EDL) were homogenized in 4 volumes of cold buffer: 50 mM Tris–HCl, 2 mM EDTA, 1% Tween 20, and 1 M NaCl, pH 6.8. Homogenates were centrifuged at 12,000*g* for 30 minutes at 4°C. AChE activity was measured by the Ellman method (Ellman et al, 1961)[5] on a Perkin-Elmer (Waltham, MA) λ25 spectrophotometer at 412 nm, 36°C, and pH 8.0.

Drugs

1,3-Bis[5(diethyl-o-nitrobenzylamino)pentyl]-6-methyluracil (C-547) was synthesized in the Institute of Organic and Physical Chemistry of the Kazan Scientific Center of the Russian Academy of Sciences. Armin (diethoxy-*p*-nitrophenyl phosphate), an inhibitor of AChE, was manufactured by the Institute of Organic Chemistry, Moscow, Russia. Neostigmine, tetraisopropylpyrophosphoramide (iso-OMPA) and the muscarinic cholinoreceptor blocker atropine were purchased from Sigma (St. Louis, MO).

Data Analysis

All results in the text and figures are expressed as mean ± SEM for n experiments. All samples were tested with Kolmogorov–Smirnov normality test. Each sample differed significantly from the normal distribution ($P < 0.05$ for every sample), so we used nonparametric tests for analysis. The effects of C-547, armin, neostigmine, iso-OMPA, and ACh on APD and CL were compared with respective basal values of APD and CL by Wilcoxon test. The effects of armin and neostigmine in the presence and absence of atropine and effects of ACh in the presence and absence of C-547 were also compared by Wilcoxon test. The effects of different concentrations of armin were compared by Mann–Whitney test because we could test only one concentration of this irreversible inhibitor in each experiment. $P \leq 0.05$ was adopted as the level of significance.

RESULTS

Modulation of APD and CL by "Classical" AChE Inhibitors

During the control period before drug application, APD50 was 19.3 ± 2.2 milliseconds, APD90 53.8 ± 4.5 milliseconds, and the CL 190.2 ± 14.0 milliseconds. The organophosphate AChE inhibitor armin (10^{-7}, 10^{-6} and 10^{-5} M) produced a marked decrease in APD50 and APD90 (Figs. 1-I, 2, 3) and an increase in CL (Figs. 2, 3). As shown in Figure 2, these effects of armin (10^{-6} M) were slowly developed, reaching the steady state after 12–13 minutes. A comparison of the APD and CL time course was similar in other AChE inhibitors, which were examined, so our discussion is limited to maximal values of AP shortening and the slowing of the firing rate. No significant changes in the membrane potential were observed in experiments with armin and all other electrophysiological experiments. The values obtained in armin experiments are shown in Figure 3.

Atropine (10^{-6} M) abolished the decrease in APD90 (Figs. 1-ID, 2, 3) and the increase in CL (Fig. 3) and also significantly reduced the decrease in APD50 induced by armin (Figs. ID, 3). Therefore, these effects are ascribed to muscarinic receptor stimulation induced by accumulation of ACh due to AChE inhibition induced by armin.[6]

Armin (10^{-5} M) altered APD and CL less than 10^{-6} M. This difference may be the result of the cholinoreceptor blocking action at high armin concentrations.[7]

The carbamate AChE inhibitor neostigmine (10^{-7}, 10^{-6}, and 5×10^{-6} M) also produced significant concentration-dependent AP shortening (Figs. 1-II, 4) and slowing of sinus rate (Fig. 4). These effects were also abolished by atropine.

Modulation of APD and CL by C-547

Our prior work has demonstrated that the application of C-547 in concentrations from 10^{-9} to 10^{-7} M increased the amplitudes and prolonged the duration of the miniatuer endplate currents in diaphragm, soleus, and EDL muscles, as is typical for AChE inhibition. This effect was maximal in soleus and EDL muscles at 5×10^{-9} M of C-547 and at 10^{-7} M in diaphragm muscles,[8] $k^0 = 2.2 \times 10^9$ mol^{-1}/min^{-1} (AChE of human erythrocytes).[1] However, in contrast to armin and neostigmine, C-547 had no significant effect on the AP configuration (Figs. 1-III, 5) and AP firing rate (Fig. 5) at all concentrations examined (10^{-9}, 10^{-8}, and 10^{-7} M).

Investigation of the BuChE Contribution to the Insensitivity of the Myocardium to C-547

BuChE is another enzyme capable of hydrolyzing ACh.[9] In contrast to skeletal muscle synapses where the contribution of BuChE to ACh hydrolysis is negligible,[10] BuChE accounts for 85%–90% of total myocardial cholinesterase activity.[11] We hypothesized that the low sensitivity of the myocardium to C-547 is due to the compensation of the deficiency of the AChE activity by BuChE. To test this hypothesis, the influence of a BuChE-specific inhibitor iso-OMPA on APD and CL in the presence of C-547 was studied. Concentrations of C-547 were selected that inhibited the AChE of skeletal muscles[8] and mammalian erythrocytes[1] but did not significantly alter APD

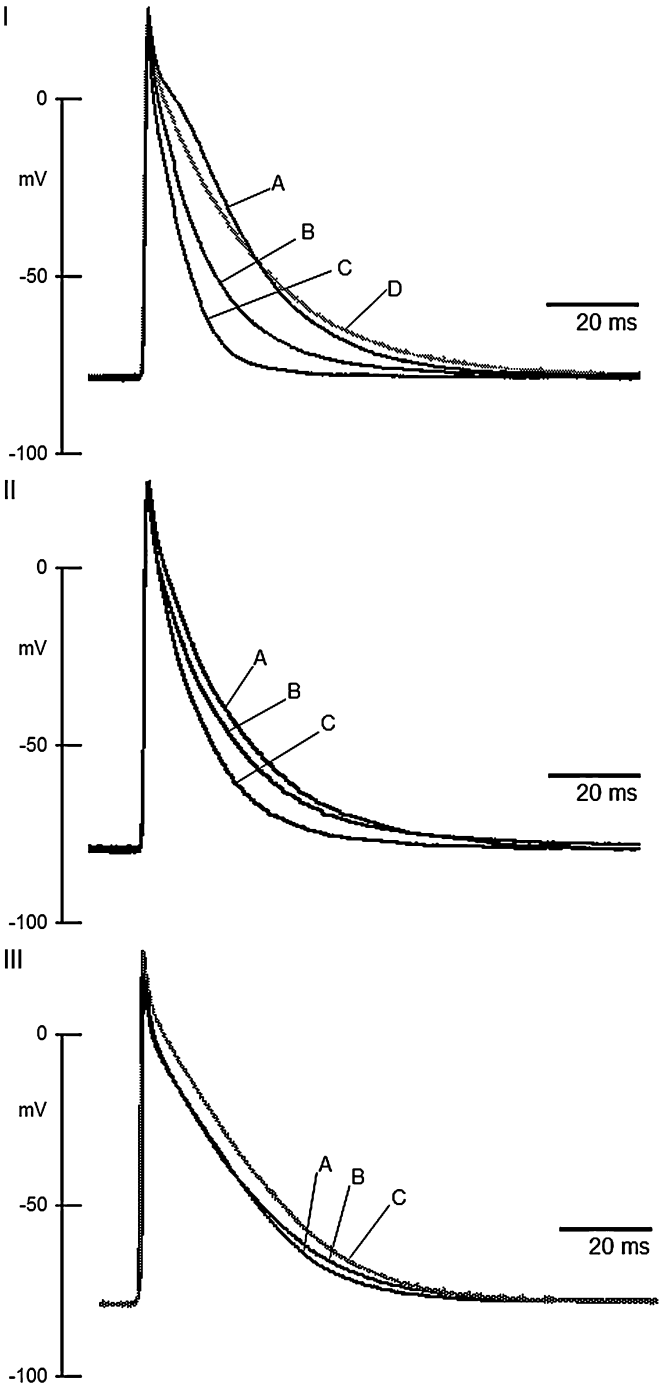

FIGURE 1. Changes in configuration of AP induced by armin (I), neostigmine (II), and C-547 (III) (original records from 3 separate experiments). I: A, control; B, 10^{-7} M armin (13th minute) C, 10^{-6} M armin (13th minute); D, recovery of initial AP duration during treatment with 10^{-6} M atropine (after treatment with 10^{-6} M armin). Membrane potential: A, −78.6 mV; B, −78.4 mV; C, −78.6 mV; D, −78.2 mV. II: A, control; B, 10^{-7} M neostigmine; C, 10^{-6} M neostigmine. Membrane potential: A, −79.5 mV; B, −78.7 mV; C, −79.5 mV. III: A, control; B, 10^{-7} M C-547; C, 10^{-5} M iso-OMPA in the presence of 10^{-7} M C-547. Membrane potential: A, −78 mV; B, −78.5 mV; C, −78.2 mV.

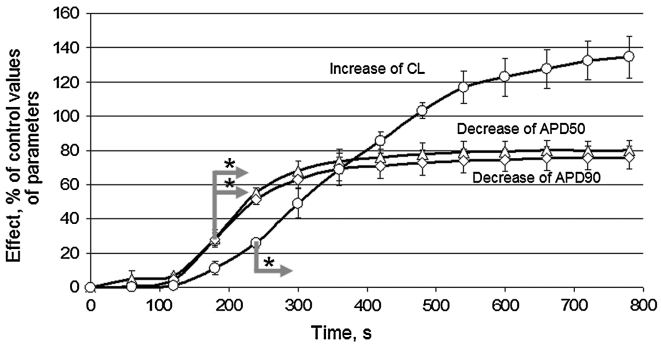

FIGURE 2. Relationship between decrease of APD and increase of CL and the time of perfusion of 10^{-6} M armin (averaged over n = 5). Control APD50, 19.9 ± 2.5 milliseconds; APD90, 52.5 ± 4.3 milliseconds; CL, 188.7 ± 17.4 milliseconds. Gray arrow with asterisks indicates the first point, where $P < 0.05$ versus the respective control values; all subsequent points are also significant.

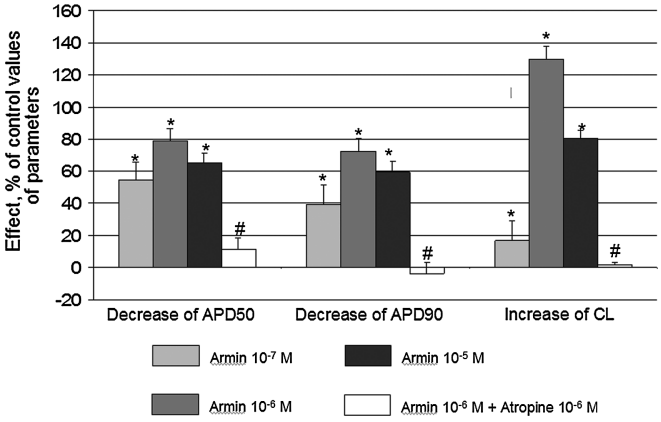

FIGURE 3. Effects of 10^{-7} to 10^{-5} M armin (n = 5 each) on AP duration and CL and influence of 10^{-6} M atropine. Ordinates: % decrease in AP duration or % increase in CL. *$P < 0.05$ versus the respective control values; #$P < 0.05$ versus 10^{-6} M armin alone.

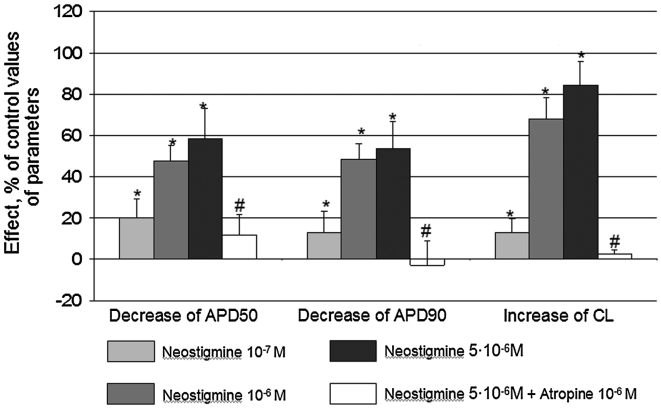

FIGURE 4. Effects of neostigmine (10^{-7} M, n = 5; 10^{-6} M, n = 5; 5×10^{-6} M, n = 4) on AP duration and CL, and influence of 10^{-6} M atropine. Ordinates: % decrease in AP duration or % increase in CL. *$P < 0.05$ versus the respective control values; #$P < 0.05$ versus 5×10^{-6} M neostigmine alone.

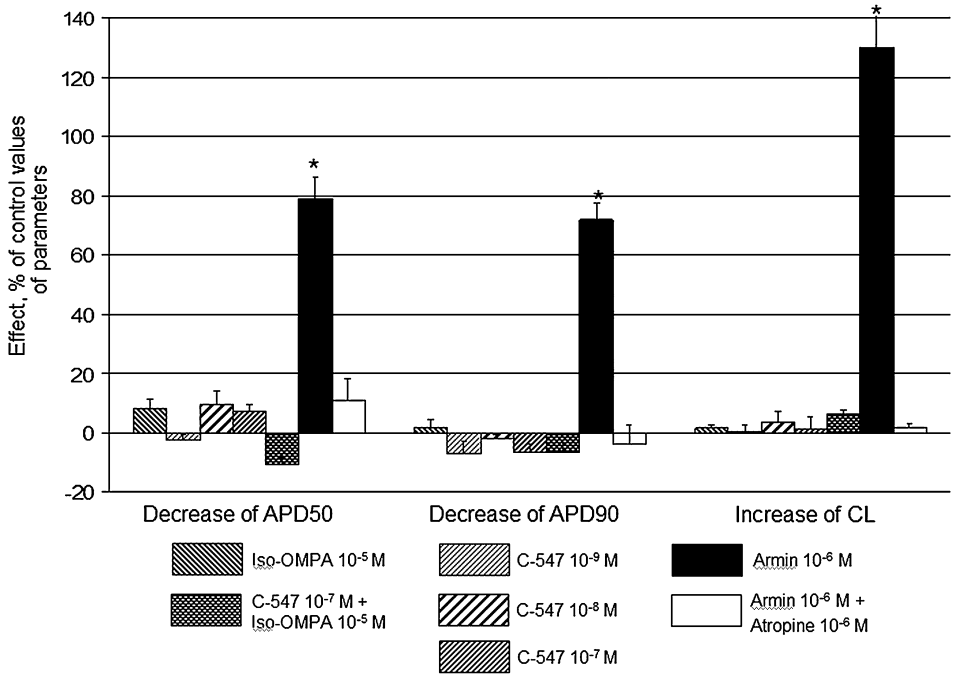

FIGURE 5. Lack of significant effects of iso-OMPA (10^{-6} M, n = 6), C-547 (10^{-9} M to 10^{-7} M, n = 6 each), and iso-OMPA (10^{-6} M) in the presence of 10^{-7} M C-547 (n = 3) in comparison with effect of 10^{-6} M armin (n = 5). Ordinates: % decrease in AP duration or % increase in CL. *$P < 0.05$ versus the respective control values.

and CL in the rat myocardium. If BuChE is capable of preventing the outcome of AChE inhibition by C-547, then iso-OMPA should cause a shortening of AP and a slowing of the sinus rate by analogy with the effects of armin and neostigmine. However, the inhibition of BuChE did not affect the AP configuration and the firing rate (Fig. 5). Thus, the low sensitivity of the heart to C-547 could not be explained by the predominant hydrolysis of ACh (secreted from parasympathetic postganglionic nerve endings) by BuChE.

Modulation of APD and CL by Exogenous ACh in the Presence of C-547

Activation of M2 muscarinic receptors leads to AP shortening and slowing of sinus rate.

We studied the alteration of APD and CL by exogenous ACh (10^{-6} M) in presence of C-547 at 10^{-8} M (Fig. 6) and compared this with the action of ACh in controls. C-547 reduced the decrease of APD50 by 57% ± 3.2%, APD90 by 67% ± 3.3% and the increase of CL by 57.3% ± 10.1% in comparison with the control effect of ACh. Thus, C-547 (10^{-8} M) reduced the effect of exogenous ACh more than 2-fold. This suggests that C-547 can block muscarinic receptors, partially or completely compensating for its anticholinesterase action.

Pharmacological Investigation of the Heart AChE Activity

Since "classical" inhibitors produce pronounced effects in the atrial myocardium, whereas C-547 does not, the presence of diffusion barriers (specific for that compound) is possible. The absence of C-547 effects in the myocardium may also be explained by insensitivity of AChE expressed in heart muscle to C-547. To test this assumption, the influence of C-547 and neostigmine on the kinetics of the reaction, which is catalyzed by AChE in homogenates of rat myocardium and EDL muscle, was studied.

The reaction speed dependence on the concentration of acetylthiocholine (ATCh) as substrate was studied at different concentrations of inhibitors. The AChE enzyme activity (1 mg of total protein) in heart and EDL muscle extracts was measured in control and in the presence of inhibitors, and the contribution of BuChE to esterase activity was excluded by the addition of a BuChE-specific inhibitor iso-OMPA to the reaction chamber. The kinetics of AChE reaction (Fig. 7A) corresponds to Michaelis-Menten, and inhibition constant K_I was determined using the Lineweaver–Burk plot (Fig. 7B). Kinetic analysis showed uncompetitive nature of AChE inhibition by C-547.

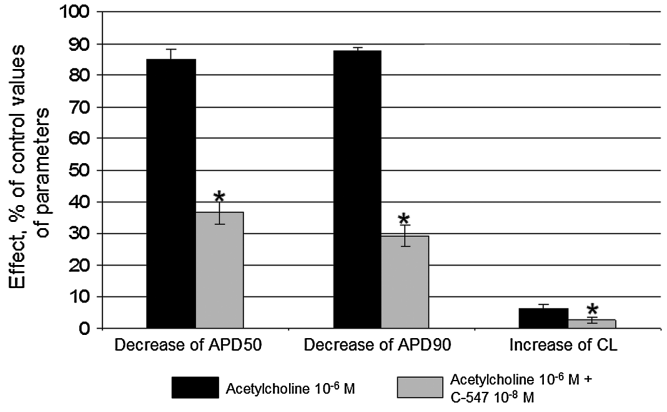

FIGURE 6. Effects of 10^{-6} M ACh in control conditions and in the presence of 10^{-8} M C-547 (n = 7). Ordinates: % decrease in AP duration or % increase in CL. *$P < 0.05$ versus 10^{-6} M ACh alone.

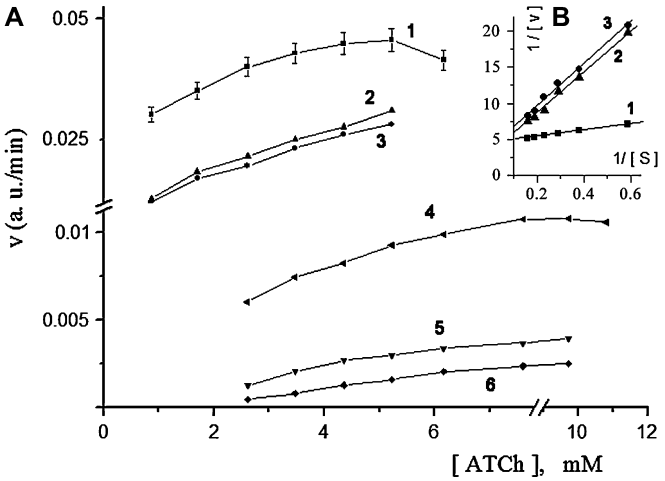

FIGURE 7. Differential inhibitory action of C-547 and neostigmine on the AChE reaction in heart and EDL muscle homogenates. A, Initial rate, *v*, of AChE reaction as a function of substrate concentration [acetylthiocholine] in the heart muscle (1–3) and EDL (4–6) at different concentrations of inhibitors: 1, control heart; 2, 0.3×10^{-4} M C-547 heart; 3, 0.6×10^{-5} M neostigmine heart; 4, control EDL; 5, 0.6×10^{-5} M neostigmine EDL; 6, 10^{-8} M C-547 EDL and 0.05 M K-phosphate, 2×10^{-5} M iso-OMPA, pH 8.0, 36°C. B, The same functions (1–3) in double-reciprocal coordinates for heart.

These data indicate that C-547 is a noncompetitive inhibitor, with a K_I (heart) = 3.6×10^{-4} M and a K_I (EDL) = 1.3×10^{-8} M. Thus, the reaction that is catalyzed by AChE in the myocardium is less inhibited by C-547 than that in EDL. Furthermore, the low sensitivity of the myocardium to C-547 revealed in electrophysiological experiments is not due to lesser accessibility of cardiac AChE as compared with that of skeletal muscle AChE. In contrast, neostigmine inhibition of the heart and skeletal muscle reaction shows nearly identical inhibition constants: K_I (heart) = 0.73×10^{-5} M and K_I (EDL) = 0.4×10^{-5} M.

DISCUSSION

Important findings in the present study are that C-547 did not significantly alter APD and CL, in contrast to the existing AChE inhibitors such as armin and neostigmine. In mammalian erythrocytes and skeletal muscles, C-547 inhibited almost completely the AChE activity in the same concentration range employed in rat atrial preparation.[1–3] Among several potential possibilities, it is highly likely that the following 2 mechanisms play important roles in the low sensitivity of the heart to C-547. First, C-547 blocks muscarinic receptors, and second, the myocardium AChE and skeletal muscle AChE have different sensitivities to C-547. Cholinolytic action has been shown for many AChE inhibitors,[12,13] but the difference between the sensitivity of myocardial AChE and that of striated skeletal muscles to inhibition has not been reported.

Thus, our results demonstrate that AChE inhibitors with high tissue selectivity may be obtained and C-547 is one such inhibitor. Tissue selectivity of this compound is based preferentially on differences between AChE in heart and skeletal muscles. Understanding of the molecular mechanisms of these differences may create the necessary prerequisites for design of heart-specific AChE inhibitors as potential cardioprotective agents.

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