# BIOPHYSICS AND BIOCHEMISTRY

# Effect of Tetraalkylammonium Derivative of 6-Methyluracil on Amplitude and Temporal Parameters of Miniature Endplate Potentials in Frog Neuromuscular Junction

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The effect of C-547, a tetraalkylammonium derivative of 6-methyluracil, a novel highly selective acetylcholinesterase inhibitor, on frog neuromuscular junction was studied. In concentrations $10^{-9}$-$10^{-7}$ M the preparation increased the amplitude and temporal parameters of miniature endplate potentials. In contrast to the effect of C-574 on purified acetylcholinesterase from mammals, the effect of this agent on frog neuromuscular junction was reversible. In a concentration of $10^{-6}$ M the preparation apart from anticholinesterase activity produced a parasympatholytic effect manifested in a decrease in amplitude and decay time constant of miniature endplate potentials accompanied by a decrease in spontaneous transmitter secretion. After washout, the parasympatholytic effect recovered more slowly, but disappeared more rapidly compared to anticholinesterase activity. These findings suggest that parasympatholytic effect of C-547 results from direct action on receptor-channel complexes in the endplate membrane.

**Key Words:** *neuromuscular junction; acetylcholinesterase inhibitors; parasympatholytic activity; pyrimidines*

Tetraalkylammonium derivatives of 6-methyluracil represent a new class of highly selective inhibitors of acetylcholinesterase (ACE, EC 3.1.1.7). These agents are characterized by inhibition rate constants $k^0 = 3.5\times10^9$ and $k^1 = 4.0\times10^8$ $M^{-1}min^{-1}$ [2,5]. In biochemical experiments, 1,3-bis[ω-(diethylorthonitrobenzilammonio)-pentyl]-6-methyluracil dibromide (C-547) irreversibly and cumulatively inhibited ACE isolated from bovine erythrocytes. Apart from high anti-ACE activity, C-547 *in vivo* demonstrates very high $LD_{50}$ to effective dose ratio (>50) [3], which is not characteristic of known cholinesterase inhibitors (organophosphorus compounds, carbamates, and tetraalkylammonium compounds). C-547 possesses a 10,000-fold higher selectivity towards ACE in comparison with that of butyrylcholinesterase (EC 3.1.1.8). The complex of C-547-ACE does not dissociate during dialysis and gel-filtration [5]. However, in contrast to mammalian ACE, the enzyme isolated from eel electric organ or cobra venom was reversibly inhibited by C-547 [2]. It was interesting to evaluate the efficiency and reversibility of the inhibitory effect of C-547 on intact membrane-bound ACE in frog neuromuscular junction (classical object in electrophysiologocal studies of the effects of ACE inactivation), where the amplitude and temporal parameters of postsynaptic sig-

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nals depend on ACE activity. Our aim was to study the effect of C-547 on the amplitude and temporal characteristics of miniature end-plate potentials (MEPP) in frog neuromuscular junction. This paper analyzes the anti-ACE effect of C-547, its dose-dependence, and reversibility of ACE inhibition.

## MATERIALS AND METHODS

Experiments were performed on neuromuscular junctions isolated from *m. sartorius* of *R. ridibunda* frogs in the autumn-winter period. The preparation was placed in a 1.7-cm$^3$ perplex chamber and perfused with Ringer solution containing (in mM): 113.0 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 3.0 $NaHCO_3$ (20.0±0.2°C, pH 7.2-7.4, 5 ml/min perfusion rate). After recording of the baseline parameters, C-547 ($10^{-9}$ to $10^{-6}$ M, A. E. Arbuzov Institute of Organic and Physical Chemistry, Russian Academy of Sciences [5]) was added to perfusion solution. Measurements were carried out for 1 h starting from the 10th min of perfusion. MEPP were recorded using standard microelectrodes filled with 2.5 M KCl (resistance 3-5 mW). MEPP were amplified and fed into a 10-bit digitizer (sampling time 10 msec). The digital signals were recorded and processed with an original software. The following parameters were analyzed: MEPP amplitude ($A_{MEPP}$), 20-80% rise time ($RT_{MEPP}$), and decay time constant ($\tau_{MEPP}$). To assess the quality of signal-noise resolution, $A_{MEPP}$ histograms were plotted fromeach series consisting of 64 signals. The series characterized by normal histograms were analyzed. The intensity of spontaneous quantal transmitter release was assessed by the interpulse periods of MEPP. The data were processed statistically using Student's *t* test ($p<0.05$) for paired samples and Microcal Origin 3.5 software.

## RESULTS

The initial steady-state membrane resting potential (MRP) in neuromuscular junction of *m. sartorius* was 57.97±2.77 mV ($n$=20). C-547 ($10^{-9}$-$10^{-6}$ M) did not

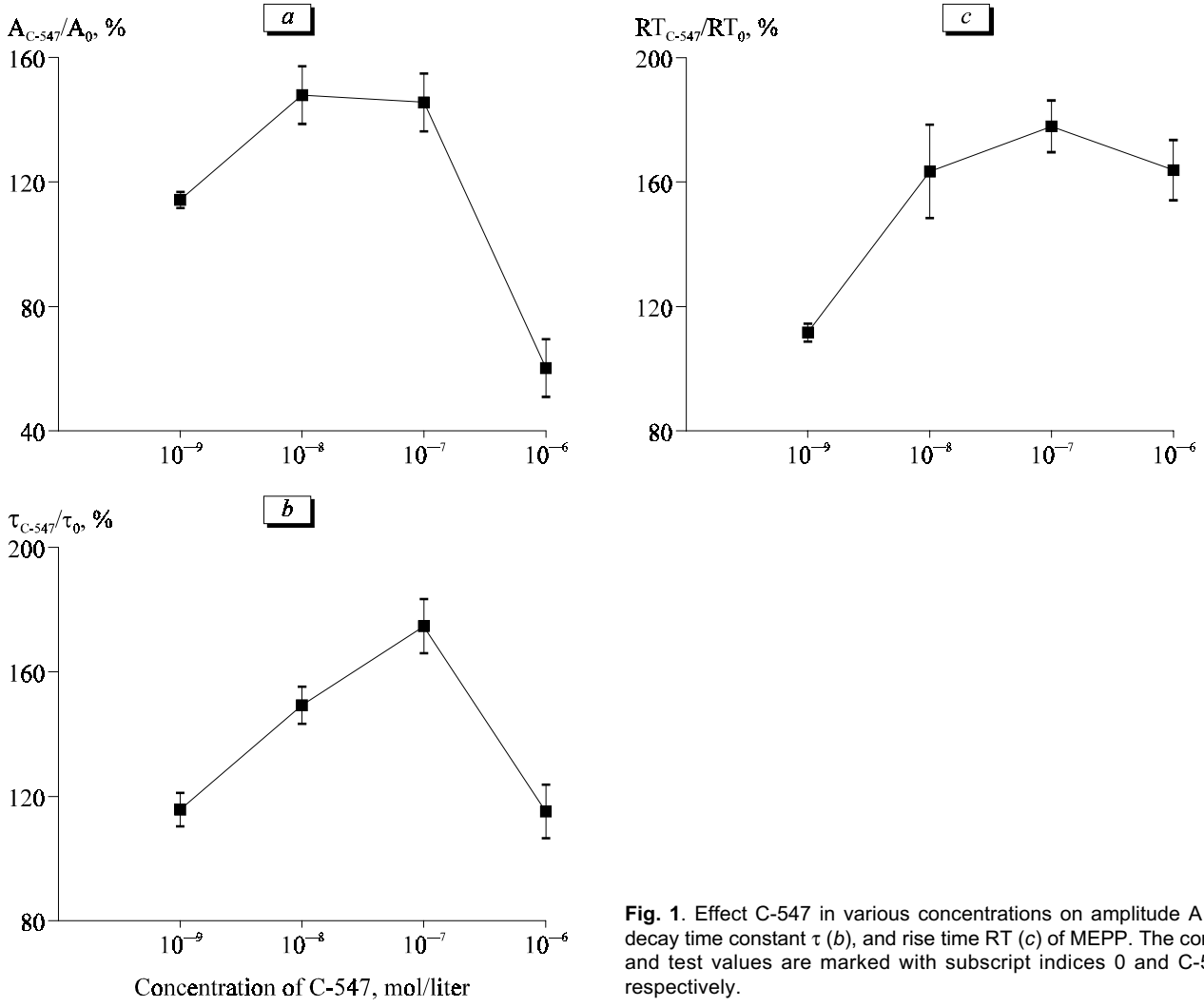


**Fig. 1**. Effect C-547 in various concentrations on amplitude A (*a*), decay time constant τ (*b*), and rise time RT (*c*) of MEPP. The control and test values are marked with subscript indices 0 and C-547, respectively.

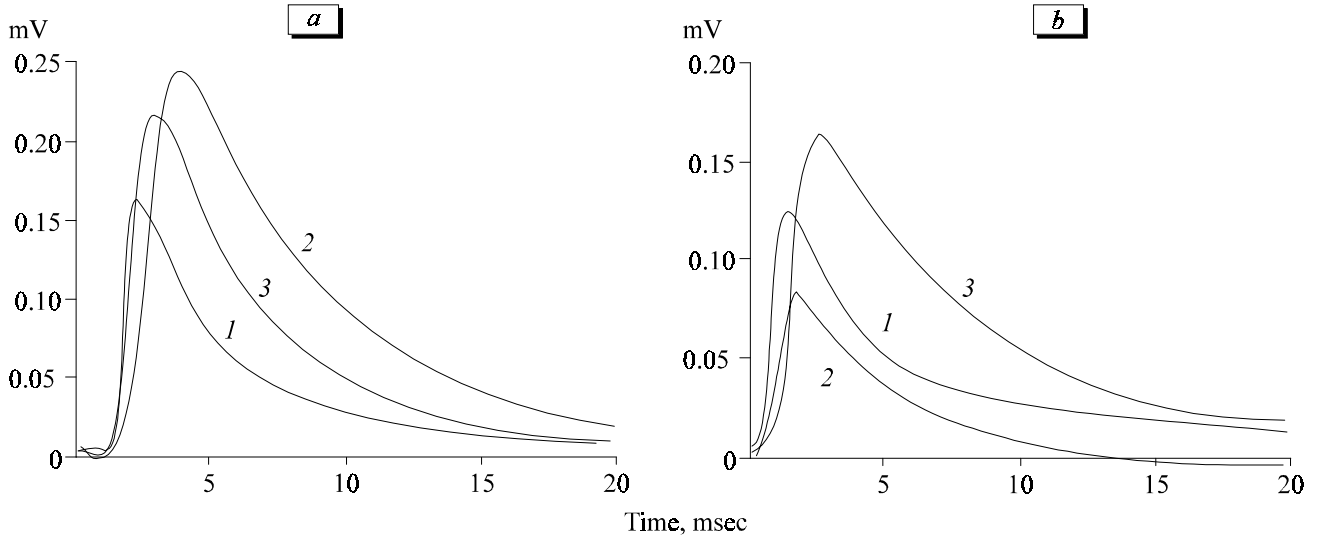


**Fig. 2.** Amplitude of MEPP in the control (*1*), after application of C-547 (*2*) in concentrations of $10^{-7}$ M (*a*) and $10^{-6}$ M (*b*), and after washout (*3*). The data are averaged over 64 MEPP.

affect MRP: 60 min after the start of perfusion it was 101±2% control value ($n$=20, $p$>0.05). This fact allowed us to consider C-547-induced changes in MEPP amplitude and temporal parameters as a result of modification of the endplate membrane and modulation of neurotransmitter secretion, rather than changes in MRP.

In the control, $A_{MEPP}$, $RT_{MEPP}$, and $\tau_{MEPP}$ were 0.15± 0.01 mV, 0.30±0.02 msec, and 3.02±0.18 msec, respectively ($n$=20). After addition of $10^{-9}$-$10^{-7}$ M C-547 the amplitude and temporal parameters of MEPP progressively increased and reached a plateau by the 60th minute (Fig. 1). Washout partially restored these parameters (Fig. 2, *a*). The observed changes in the amplitude and temporal parameters of MEPP attest to anti-ACE activity of C-547. However, partial reversibility of the effect does not agree with biochemical data obtained on ACE isolated from bovine erythrocytes [5]. Reversibility of the effect of C-547 on ACE in neuromuscular junction was studied in experiments with a long-term (3.5 h) washout. Since long-term MEPP recording in a single muscle fiber can be accompanied by a decrease in MRP and the corresponding changes in MEPP parameters, in this experimental series MEPP were measured in 15 control muscle fibers, then C-547 ($10^{-7}$ M) was applied for 1 h, after which the preparation was washed for 3.5 h with initial Ringer solution. Washout reduced all test parameters: $A_{MEPP}$ decreased from 140±12 to 113±14%, $RT_{MEPP}$ from 192±14 to 96±7%, and $\tau_{MEPP}$ from 139±4 to 92±6% ($n$=15, $p$>0.05 compared to initial control values). Thus, the anti-ACE effect of C-547 in frog neuromuscular junction is reversible.

As was previously shown, C-547 in concentrations below $10^{-7}$ M demonstrated anti-ACE activity, but increasing the concentration to $10^{-6}$ M was accompanied by a decrease in $A_{MEPP}$ and $\tau_{MEPP}$ (Fig. 1 and 2, *b*). Figure 3 shows the time course of the effects of C-547 ($10^{-6}$ M) in a representative experiment: during the first 15-20 minutes after application $A_{MEPP}$, $RT_{MEPP}$, and $\tau_{MEPP}$ increased (anti-ACE affect) , but then $A_{MEPP}$ and $\tau_{MEPP}$ decreased (parasympatholytic activity), while $RT_{MEPP}$ remained elevated (anti-ACE effect is still present). Thus, in this concentration C-547 demonstrated both ACE inhibiting and parasympatholytic activity. Parasympatholytic effect developed more slowly than anti-ACE activity, but disappeared more rapidly than the anti-ACE effect.

The changes in spontaneous quantal secretion of acetylcholine induced by C-547 in concentrations of $10^{-9}$-$10^{-6}$ M, which were revealed by the analysis of interpulse intervals, showed that when applied in the concentrations of $10^{-9}$-$10^{-7}$ M, C-547 produced no significant changes in the distribution of interpulse intervals and MEPP mean frequency ($\varphi_{MEPP}$). However, increasing the concentration of C-547 to $10^{-6}$ M led to a decrease in $f_{MEPP}$ by 42±5% in comparison with the initial value (0.71±0.24 Hz, $n$=5, $p$<0.05). This decrease was not accompanied by changes in the distribution of MEPP interpulse intervals. After 60-min washout with control Ringer solution, $\varphi_{MEPP}$ was 99± 19% of the control value. Therefore, C-457 in a concentration of $10^{-6}$ M demonstrated anti-ACE and parasympatholytic effects, and reversibly decreased the rate of spontaneous quantal release of acetylcholine from frog nerve motor terminals.

Thus, C-547 demonstrated anti-ACE activity and increased the amplitude and temporal parameters of MEPP even in nanomolar concentrations. However, in contrast to data indicating on slowly formed and tightly bound complex of C-547 with purified ACE isola-

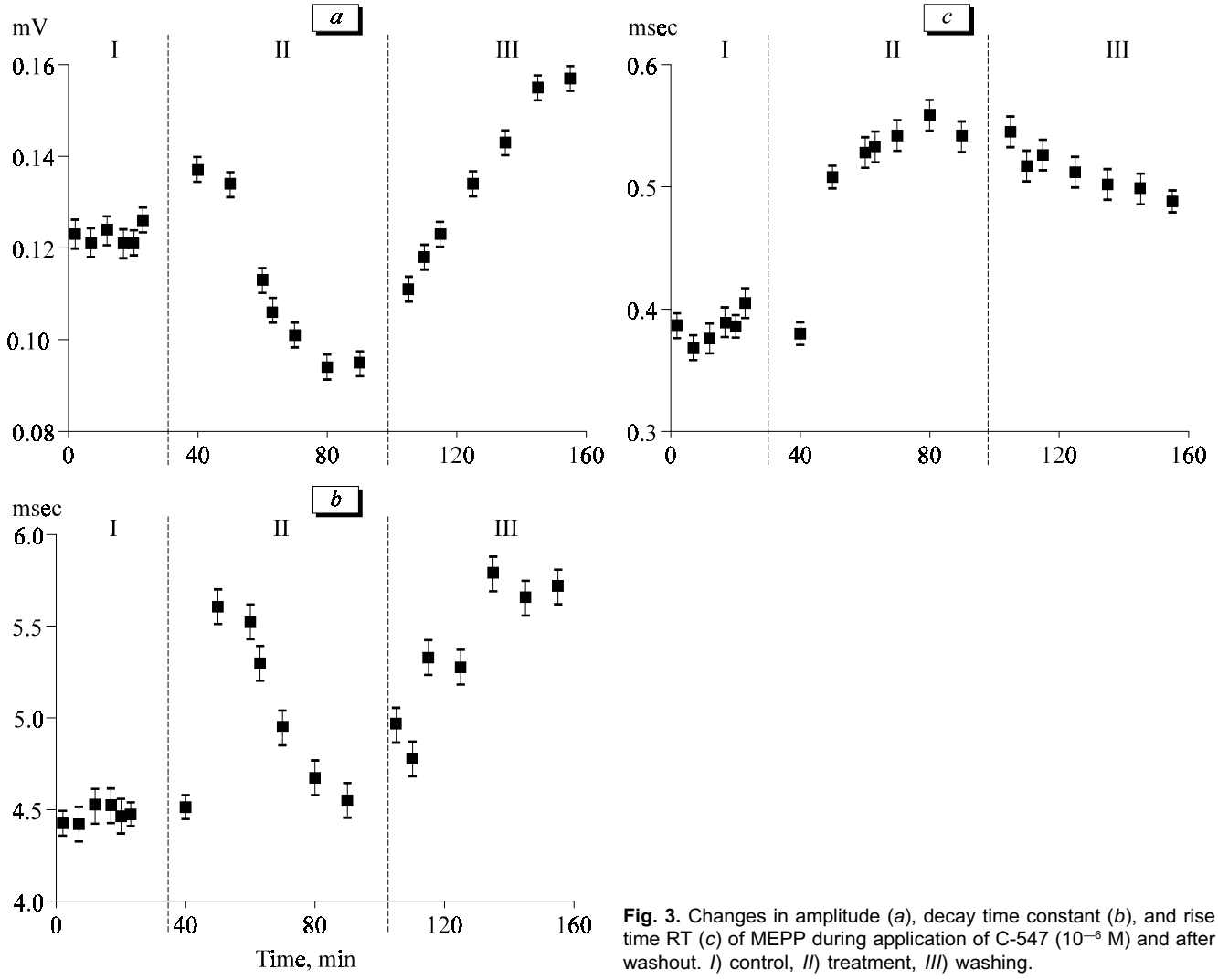


**Fig. 3.** Changes in amplitude (*a*), decay time constant (*b*), and rise time RT (*c*) of MEPP during application of C-547 ($10^{-6}$ M) and after washout. *I*) control, *II*) treatment, *III*) washing.

ted from bovine erythrocytes [5,8,9], interaction of C-547 with intact synaptic ACE in the frog neuromuscular junction was reversible. Probably, this discrepancy attests to significant differences in molecular structure of membrane-bound ACE in cold-blooded animals and purified ACE from mammals. These differences probably involve the structure of nucleotide (pyrimidine) recognizing sites of the enzyme [2,4,5]. In purified ACE from bovine erythrocytes these sites coincide with the location of 6-methyluracil fragment relatively to the tetraalkylammonium groups, which results in very high selectivity and irreversibility of binding despite the absence of covalent bonds [1,6,9]. The absence of such complementation in ACE macromolecules from frog neuromuscular junction determines reversibility of C-547 binding with the enzyme. Reversibility of C-547 binding demonstrated for ACE from eel electric organ or common cobra venom also results from peculiarities in the structure of the active sites of these enzymes [2].

C-547 in a concentration of $10^{-6}$ M modulated spontaneous quantum release of neurotransmitter from motor nerve terminals, which lead to a decrease in the mean frequency of MEPP. This effect can result from a direct effect of C-547 or from the effect of endogenous acetylcholine; there is evidence that parasympathomimetics inhibit spontaneous secretion of neurotransmitter in frog neuromuscular junction [7]. However, inability of C-547 to affect the frequency of MEPP in concentration of $10^{-7}$ M producing maximum anti-ACE effect approximately equal to that observed at $10^{-6}$ M, led us to a conclusion that inhibition of spontaneous neurotransmitter release and parasympatholytic activity are mediated by a direct effect of C-547 on motor nerve endings.

## REFERENCES

1. A. Albert, *Selective Toxicity* [in Russian], Vol. 2, Moscow (1989), pp. 102-103.

2. K. A. Anikienko, E. A. Bychikhin, V. K. Kurochkin, *et al., Dokl. Ross. Akad. Nauk*, **376**, No. 6, 818-822 (2001).
3. M. Dyxon and E. Webb, *Enzymes* [in Russian], Moscow (1966).
4. A. S. Polezina, K. A. Anikienko, and V. K. Kurochkin, *Ross. Khim. Zh.,* **43**, No. 5, 72-79 (1999).
5. V. S. Reznik, K. A. Anikienko, V. K. Kurochkin, *et al., Dokl. Ross. Akad. Nauk*, **362**, No. 1, 68-70 (1998).
6. A. G. Karczmar (Ed.), *IEPT. Anticholinesterase Agents,* **1**, Sect. 13, 68-69 (1970).
7. E. Nikolsky, E. Bukharaeva, E. Strunsky, and F. Vyskocil, *Br. J. Pharmacol.,* **104**, 1024-1032 (1991).
8. D. V. Santi and G. L. Kenyon, in: *Approaches to the Rational Design of Enzyme Inhibitors. Burgers Medicinal Chemistry*, Ed. M. E. Wolff, New York (1980), pp. 349-391.
9. M. Whittaker in: L. Beckman (Ed.), *Human Genetics,* Vol. 11, Basel (1986), pp. 18-29.