QUANTITATIVE ESTIMATION OF SYNAPTIC ACETYLCHOLINESTERASE INHIBITION BY GALANTHAMINE USING MINIATURE END-PLATE CURRENT PARAMETERS

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Acetylcholinesterase (AChE) inhibitors are widely used in medicine and agriculture and as a tool to investigate cholinergic synaptic transmission [2]. The kinetics of inhibition of the purified enzyme and of AChE in tissue homogenates has been well studied. However, it is not yet absolutely clear how native AChE, localized in the synaptic cleft and responsible for normal functioning of the cholinergic synapse, is inhibited. This can be explained mainly by the absence of a reliable method of determining activity of natural synaptic (functional) AChE [7].

The amplitude and duration of miniature end-plate currents (MEPC) arising in response to release of single quanta of acetylcholine (ACh) from the nerve ending, are determined by the kinetics of opening and closing of ionic channels and the velocity of ACh hydrolysis in the synaptic cleft [5]. Definite correlation ought to exist between the degree of inhibition of AChE and the increase in amplitude and duration of single-quantum responses [1, 7], and it was accordingly decided to use this for quantitative estimation of inhibition of synaptic AChE.

EXPERIMENTAL METHODS

Experiments were carried out on phrenic nerve–diaphragm preparations of albino rats in a chamber containing continuously flowing solution, saturated with carbogen (95% O_2 + 5% CO_2). The composition of the solution (in mM) was: NaCl – 137; KCl – 5; $CaCl_2$ – 2; $MgCl_2$ – 2; $NaHCO_3$ – 24; NaH_2PO_4 – 1; glucose – 11; pH 7.4-7.5; T 28°C. To obtain different degrees of inhibition of AChE galanthamine (a tertiary ammonium compound and competitive inhibitor of AChE of reversible type) was used in various concentrations.

MEPC was recorded intracellularly by the voltage clamp method using two microelectrodes, starting from the 30th minute of action of galanthamine. The membrane potential of all the fibers was clamped at –100 mV. The amplitude and half-decay time ($T_{1/2}$) of MEPC were measured.

EXPERIMENTAL RESULTS

The higher the concentration of galanthamine, i.e., the higher the degree of inhibition of AChE, the greater the increase in amplitude and duration of MEPC (Table 1). Let us attempt to establish the character of the relationship between the amplitude of MEPC and synaptic AChE activity.

The density of active centers of ACh receptors (AChR) is such (~$2 \cdot 10^4$ μ^{-2} [6]) that a quantum containing about 10^4 ACh molecules is bound on a postsynaptic area of 0.3-0.5 μ^2 [1, 6, 9]. In that case 1500-3000 ionic channels are opened [1, 8, 9] and an MEPC with an amplitude of several nanoamperes is generated. Since the "radius of action" of the quantum is 300-400 nm and the width of the synaptic cleft about 50 nm, the binding zone of the quantum can be represented as a very thin disk. After release of a quantum, ACh molecules therefore bind with the AChR, not at once, but in the course of diffusion along the synaptic cleft. Under these circumstances some ACh molecules are hydrolyzed by synaptic AChE. When AChE is inhibited these losses are reduced and the number of ACh molecules reaching the AChR, i.e., the amplitude of MEPC, increases.

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TABLE 1. Parameters of MEPC in Control and during Action of Galanthamine (M ± m)

Parameters of MEPC	Galanthamine concentration, M				
	0	$0,86 \cdot 10^{-7}$	$2,7 \cdot 10^{-7}$	$8,6 \cdot 10^{-7}$	$27 \cdot 10^{-7}$
Amplitude, nA	6,14±0,22	6,65±0,38	7,65±0,26	7,82±0,40	8,55±0,40
A_i/A_o	1	1,08±0,07	1,25±0,06	1,27±0,08	1,39±0,06
Half-decay time, msec	0,56±0,02	0,66±0,02	0,80±0,02	1,04±0,05	1,49±0,08
Number of fibers investigated	34	19	17	24	21

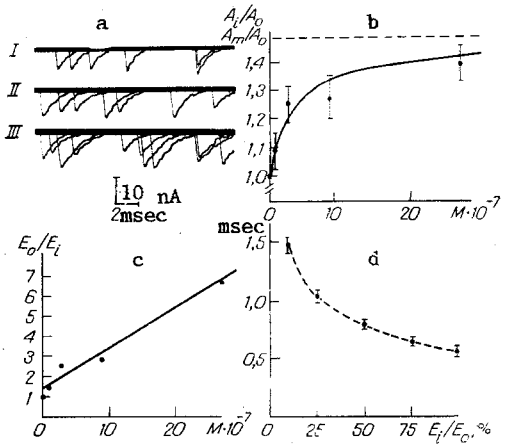

Fig. 1. Parameters of MEPC and activity of synaptic AChE during action of galanthamine. a) Trace of MEPC in control (I) and under influence of galanthamine in concentrations of $2.7 \cdot 10^{-7}$ M (II) and $27 \cdot 10^{-7}$ M (III); b) change in amplitude of MEPC (A_i/A_0) depending on galanthamine concentration. Curve drawn on the basis of equation (6) for A_0 = 6.14 nA, A_m = 9.16 nA, and $K_i = 2.8 \cdot 10^{-7}$ M; c) relationship between value of E_0/E_i calculated from equation (3) and galanthamine concentration. Straight line plotted by method of least squares; d) value of $T_{1/2}$ of MEPC for different degrees of inhibition of AChE by galanthamine. Explanation in text.

The fraction of ACh molecules in the quantum that bind with AChR depends in the general case on the velocity constants of binding with AChR and AChE, on concentrations (densities) of the reacting molecules, and on the rate of diffusion [8]. Thanks to the high density of AChR, binding of the quantum takes place on a small area under conditions of a saturating ACh concentration [6, 9]. The fraction of ACh molecules remaining free or leaving the cleft due to diffusion, according to data of mathematical modeling, is small (about 10%) [9]. Considering these circumstances, it can be postulated that virtually all the ACh molecules in the quantum are bound either with AChR or with AChE, and in a ratio determined by the densities of distribution of these macromolecules. In that case, as a first approximation, we can write:

$$A_0 = \frac{A_m R}{R + E_0}, \quad (1)$$

$$A_i = \frac{A_m R}{R + E_i}, \quad (2)$$

where A_0 and E_0 represent the amplitude of MEPC and the density of free AChE in the absence of inhibitor respectively, A_i and E_i represent the same at a given degree of inhibition of AChE, A_m is the greatest possible (mean) amplitude of MEPC provided that all ACh molecules in the quantum bind with AChR; R denotes the density of free AChR. It follows from equations (1) and (2) that:

$$\frac{A_i}{A_0} = \frac{1}{A_0/A_m + (1 - A_0/A_m)\, E_i/E_0}. \quad (3)$$

In the absence of rhythmic activity in the synaptic cleft a steady state is established in the enzyme–substrate–inhibitor system, and can be described in the case of action of a competitive inhibitor of reversible type by the Scheme [3]:

$$\begin{aligned} E + S &\rightleftharpoons ES \longrightarrow E + P \\ E + I &\rightleftharpoons EI, \end{aligned} \quad (4)$$

where E denotes enzyme and S substrate (ACh); I the inhibitor; P the reaction product; ES the enzyme–substrate complex, and EI the enzyme–inhibitor complex. In accordance with the Scheme [4]:

$$\frac{E_i}{E_0} = \frac{K_m K_i + K_i [S]}{K_m K_i + K_i [S] + K_m [I]} = \frac{1}{1 + C [I]}, \quad (5)$$

where K_m is the Michaelis constant for the enzyme–substrate reaction; K_i the inhibitory constant; $C = K_m/K_i\ (K_m + [S])$. In the steady state the ACh concentration in the synaptic cleft is mainly determined by the constant release of ACh from the nerve ending in nonquantal form and it is of the order of 10^{-8} M [4]. Since K_m is of the order of 10^{-4} M [7], it follows that $[S] \ll K_m$ and $C \simeq 1/K_i$.

During release of a quantum the local ACh concentration in the synaptic cleft rises sharply to 10^{-3} M [6]. It can be tentatively suggested, however, that in a short time of quantum binding (about 100 microseconds) ACh, even in that concentration, cannot displace the inhibitor from the EI complex, and the rate E_i/E_0 is unchanged. Having accepted this assumption, by substituting (5) in (3), we obtain:

$$\frac{A_i}{A_0} = \frac{1 + C [I]}{1 + A_0 C [I]/A_m}. \quad (6)$$

It follows from equation (6) that with an increase in [I] the value of A_i/A_0 ought to approximate asymptotically to the value of A_m/A_0. The shape of the experimental dependence obtained (Fig. 1b) coincides with that predicted. By the method of least squares, using equation (6) as an approximating function, the values of A_m = 9.16 nA and $K_i = 2.8 \cdot 10^{-7}$ M were obtained from the experimental data. Closely similar values of the inhibitory constant of galanthamine have been obtained by biochemical methods on erythrocytes [2].

We know from enzyme kinetics [3] that in the steady state the ratio E_0/E_i (and also the ratio between the corresponding velocities of the enzyme reaction) during competitive inhibition ought to be a linear function of the concentration of inhibitor (see also equation 5). The dependence of E_0/E_i on galanthamine concentration, calculated by equation (3) on the basis of experimental data on changes in the amplitude of MEPC, was in fact found to be close to linear: coefficient of correlation $r = 0.989$, $p < 0.01$ (Fig. 1c). This result can be regarded as proof of the adequacy of the suggested model.

In the modern view, in the presence of completely active AChE the "spent" ACh is removed from the synaptic cleft by hydrolysis, and decay of MEPC reflects mainly the rate of opening of ionic channels [5]. On inhibition of AChE removal of ACh from the cleft and decay of MEPS take place more slowly, and it is important to establish the type of dependence of the degree of inhibition of AChE on $T_{1/2}$ of MEPC. To do this, we can calculate by equation (5) the value of E_i/E_0 (at $K_i = 2.8 \cdot 10^{-7}$ M) for different galanthamine concentrations and we can use the data of the increase in $T_{1/2}$ under these conditions (Table 1). The relationship thus plotted (Fig. 1d) coincides with that obtained by mathematical modeling of the factors determining the time course of single-quantal responses [1].

The suggested model is constructed, of course, on some degree of simplification of processes actually taking place in the synaptic cleft under the influence of ACh quanta, but it has the advantage that it enables the numerical values of the densities of AChR and AChE and the velocity constants of the reactions, which are not accurately known, to be dispensed with. It is to be hoped that further fruitful development of the electrophysiological method of quantitative estimation of synaptic AChE activity and of the effectiveness of its inhibitors will take place.

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ASYMMETRY OF LIPIDS AND α-TOCOPHEROL DISTRIBUTION IN THE OUTER AND INNER MONOLAYER OF BILAYER LIPID MEMBRANES

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Ever-increasing attention is being paid to the targeted transport of drugs in the patient [4-6]. As a rule the agents used to carry drugs (antibiotics, corticosteroids, insulin, cytotoxic substances, etc.) to organs and target cells, are artificial phospholipid vesicles or liposomes, which are biologically inert and completely biodegradable [6]. The efficiency of delivery of the drugs depends on the duration of circulation of the liposomes and of preservation of their integrity in the blood stream, which may be disturbed as the result of interaction of the outer monolayer of the liposomes with various blood components (acyl hydrolases, acyl transferases, lipoproteins, macroglobulins, etc.) [5, 6]. Stabilization of liposomes by α-tocopherol (TP), a natural membrane protector, may be a promising method of obtaining long-term liposomal preparations. TP also is used independently as an effective therapeutic agent [4, 8, 9]. Meanwhile it has been shown that TP has limited ability to insert itself into phospholipid vesicles and a low rate of transbilayer migration [7, 9].

The aim of this investigation was to study the distribution of TP between the outer and inner monolayers of synthetic phospholipid liposomes, a matter of the utmost importance when ways of saturating biological structures with TP are contemplated.

EXPERIMENTAL METHODS

Liposomes were prepared from dioleoylphosphatidylcholine and phospholipids of the plasma membranes of rat brain synaptosomes. The liposomes were obtained by evaporation of phospholipids under argon, followed by shaking in Tris-HCl buffer (Tris-HCl 50 mM, NaCl 100 mM, pH 7.4) or bicarbonate buffer ($NaHCO_3$ 120 mM, NaCl 100 mM, pH 8.2), and treatment on a UZDN-2 ultrasonic disintegrator (22 kHz), and repeated sonication (12 × 15 sec, with intervals of 30 sec) at 0-4°C until complete clarification of the liposomal suspension. To reduce destruction of lipids, the membranes were disintegrated in a cylindrical resonator, excluding any direct contact between resonator and liposome solution. The liposomes thus obtained were centrifuged at 90,000g for 20 min, and supernatant containing monolayer liposomes was used for the measurements [10]. To incorporate TP into the outer and inner monolayers of liposomes, solutions of phospholipids and TP in chloroform were mixed in definite proportions. The mixture was evaporated to dryness in a current of argon and liposomes prepared from it. The fluorescence spectra of TP were recorded on Hitachi MPF-2A and SPF-850 spectrofluorometers (Japan) in a 1-cm cuvete using slits 5 nm wide. The TP was oxidized with potassium ferricyanide. To determine the localization of amine-containing phospholipids of phospha-

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