Phenazepam in Therapeutic and Ultralow Doses *in Vitro* Modulates the Content of Lipid Peroxidation Products and Acetylcholinesterase Activity in Membrane Fraction from Mouse Brain

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In vitro incorporation of tranquilizer phenazepam in a concentration of 10^{-13} M into membrane fraction from mouse brain produced a prooxidant effect. In concentrations of 10^{-5}-10^{-9} and 10^{-15}-10^{-17} M this agent possessed antioxidant activity. Phenazepam significantly decreased the maximum rate of enzymatic reactions (10^{-5} and 10^{-15} M) and Michaelis constant (10^{-5} M) for acetylcholinesterase. Incorporation of phenazepam in ultralow doses into membrane modified its lipid components (estimated by lipid peroxidation) and functional state, and this effect was comparable with the influence of this substance in standard doses. This probably contributes to the physiological effect of ultralow doses of phenazepam in.

Key Words: *acetylcholinesterase; benzodiazepines; biological membranes; lipid peroxidation; ultralow concentrations*

Nowadays, stress loads on humans constantly increase. The unstable economic and political situation, uncertainty in the future, information overload, and other factors cause severe neurotic disorders. This determines increased consumption of tranquilizers, the drugs reducing anxiety, fear, and agitation. Benzodiazepines are most widely used tranquilizers. The effective and inexpensive preparation phenazepam (7-bromo-5-(2-chlorophenyl)-1,2-dihydro-3H-1,4-benzodiazepine-2-on) is the most popular anxiolytic drug. In clinical practice phenazepam is prescribed in doses of 10^{-6}-10^{-5} M, in experiments this substance is used in concentrations of 10^{-7}-10^{-5} mol/kg body weight.

Similarly to most tranquilizers, phenazepam produces various side effects (*e.g.*, myorelaxant, sedative, hypnotic, and amnesic action); moreover, phenazepam can produce damage to the liver [5]. The search for new tranquilizers is in progress.

A promising approach is the use of classical tranquilizers in ultralow concentrations (some orders of magnitude lower than standard doses). Routine tests showed that phenazepam in doses of 10^{-10}-10^{-11} mol/kg produces a potent anxiolytic effect [2]. It should be emphasized that anxiolytic activity of phenazepam surpasses that of daytime tranquilizers. Phenazepam in ultralow concentrations produces no side effects observed after treatment with this agent in a dose of 10^{-7} mol/kg or higher [2].

Pharmacological effect of benzodiazepines is realized via their interaction with allosteric sites in $GABA_A$ receptors (subtypes $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$), which modulates the rate of opening of chlorine channels in these receptors [3]. The composition and structure of the lipid bilayer and intensity of lipid peroxidation (LPO) in neuronal membranes play an important role in functional activity of neurotransmitters (*e.g.*, GABAergic system) and relationships between neuromodulators [4]. LPO determines the functional state of the lipid bilayer, since it is associated with other characteristics of membrane lipids in the general regulatory system [1].

Phenazepam in standard doses modulates the intensity of LPO *in vitro* and *in vivo* [3,6]. Here we studied *in vitro* effects of phenazepam in various concentrations (including ultralow doses) on LPO in brain membranes. Functional state of membranes was estimated by activity of membrane-bound acetylcholinesterase (AChE).

MATERIALS AND METHODS

Experiments were performed on outbred SHK mice. For isolation of membrane fraction unpurified synaptosomes obtained by differential centrifugation was

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treated by hyposmotic shock. Protein content was measured by the method of Lowry [12]. Lipids were extracted by the method described previously [8] with modifications. The content of lipid hydroperoxides (conjugated dienes) was estimated by UV spectrophotometry [13]. Peak optical densities D_{205} and D_{230} were determined at D_{205} 0.5-0.7 optical density units. In this range the intensity of absorption is proportional to lipid concentration. The content of lipid hydroperoxides was estimated by the D_{230}/D_{205} ratio (A_2/A_1) calculated for the control and experimental samples at the same D_{205}.

For incorporation of phenazepam into membranes, their suspension was incubated with phenazepam dissolved in ethanol to concentrations of 10^{-17}, 10^{-15}, 10^{-13}, 10^{-11}, 10^{-9}, 10^{-7}, 10^{-5}, and 10^{-4} M at 4°C for 20 h. Ethanol concentration in control and experimental samples did not exceed 0.5%. AChE activity was measured spectrophotometrically using acetylcholine iodide (Sigma) as the substrate [11]. The kinetics for formation of the reaction product was recorded continuously on a DU-50 spectrophotometer (Beckman). Kinetic parameters of the enzymatic reaction, Michaelis constant (K_M) and maximum rate (V_{max}), were determined from the substrate dependence of the initial rate (as parameters for the Michaelis-Menten equation). We performed 4-5 parallel measurements of the initial rate for each sample at various concentrations of the substrate.

The results were analyzed by Student's *t* test.

RESULTS

Phenazepam in concentrations of 10^{-5}-10^{-9} M produced an antioxidant effect. The content of primary oxidation products in membranes treated with phenazepam decreased by 25-30% compared to the control (Fig. 1). After treatment with 10^{-15} and 10^{-17} M phenazepam the content of LPO products also tended to decrease (antioxidant effect). At the same time phenazepam in a dose of 10^{-13} M almost 2-fold increased the content of conjugated dienes compared to the control, which suggests that this concentration intensified LPO in brain membranes. Our results suggest that phenazepam in the ultralow concentration range produces a dual effect on LPO (similarly to its influence in standard doses) intensifying or inhibiting LPO depending on its concentration.

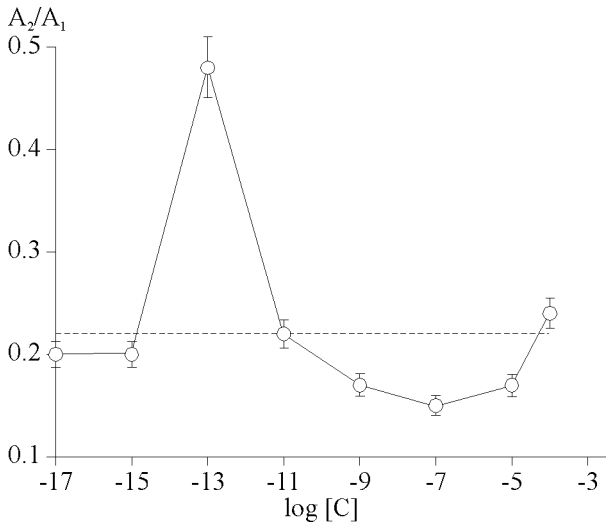

Fig. 1. Content of conjugated diene (A_2/A_1) in lipids of mouse brain membranes after *in vitro* treatment with phenazepam in different concentrations (log [C]). Dotted line: concentration of conjugated dienes in the control (without phenazepam).

The mechanisms of changes in LPO produced by phenazepam in ultralow doses remain unclear. It can

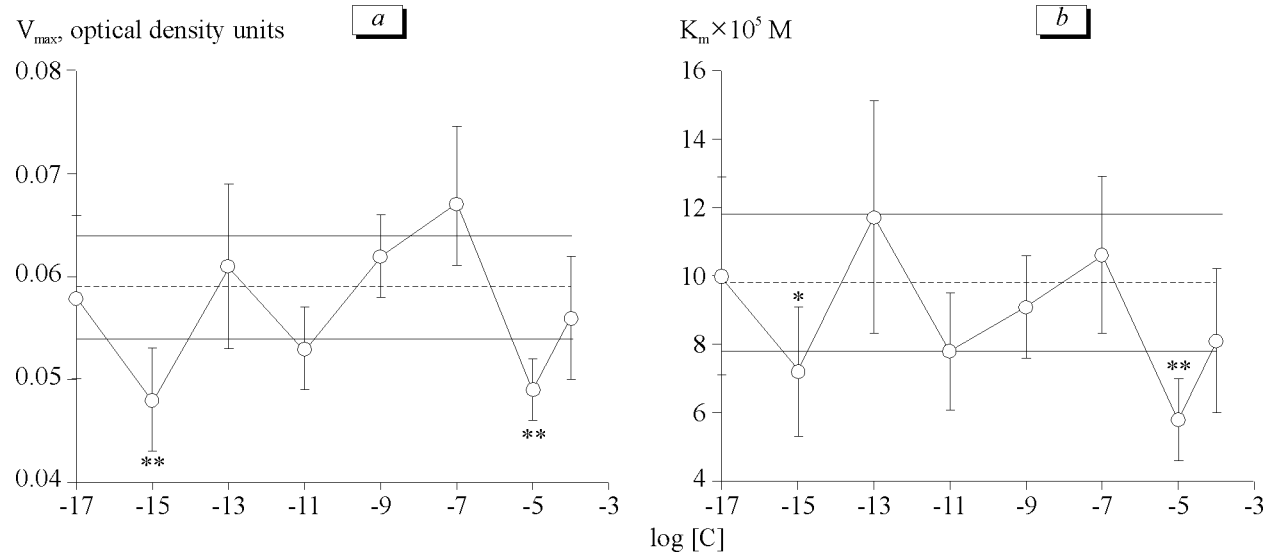

Fig. 2. Kinetic parameters for membrane-bound acetylcholinesterase after *in vitro* treatment with phenazepam in various concentrations (log [C]): maximum reaction rate (V_{max}, *a*) and Michaelis constant (K_M, *b*). **p*<0.01 and ***p*=0.07 compared to the control.

be hypothesized that changes in LPO intensity are mediated by interaction with putative high-affinity receptors on the membrane. Different pharmacological effects of various preparations in the standard dose range are mediated by their binding to benzodiazepine receptors characterized by different affinity [9,13]. The existence of receptors for ultralow concentrations of ligands cannot be excluded.

Activity of membrane-bound AChE reflects structural and functional state of cell membranes. We studied the effects of *in vitro* incorporation of phenazepam in various concentrations (including ultralow doses) into brain membranes. Significant changes in kinetic parameters of AChE were observed after treatment with phenazepam in concentrations of 10^{-5} and 10^{-15} M (Fig. 2).

Our results suggest that phenazepam in ultralow concentrations modifies lipid components and functional state of membranes, and this effect is comparable with the influence of this substance in standard doses. These changes probably contribute to the physiological effect of phenazepam. Our findings substantiate the possibility of using phenazepam in ultralow doses in clinical practice.

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