Effect of Diazepam on the Transmembrane Potential and Physicochemical State of the Thymocyte Membrane in Rats during Exposure to Cold

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Exposure of restrained rats to cold caused a drop in rectal temperature by 3°C, attenuated membrane potential by $\Delta\psi_T$, decreased the level of lipid peroxidation products, and increased the viscosity of membrane lipids in thymocytes. Although diazepam (5 mg/kg, 1 h prior to experiment) decreased $\Delta\psi_T$ and lipid viscosity under comfortable temperature, it prevented the cold-induced changes in these parameters. Incubation of intact rat thymocytes with diazepam (0.2 μg/ml) decreased $\Delta\psi_T$, increased lipid viscosity, and did not change the intensity of lipid peroxidation. Possibilities are discussed to reduce with the help of diazepam the cold-related energy losses not only due to its effect on the central benzodiazepine receptors, but also due to changes in functional and structural parameters of the membranes caused by interaction with peripheral receptors.

Key Words: *cold; diazepam; thymocyte membrane; transmembrane potential; lipid peroxidation*

There is evidence that pretreatment with benzodiazepines decreases thermal losses induced by moderate cold [2,11]. This effect is related to the inhibition of stress symptoms by these drugs, primarily due to their interaction with the central benzodiazepine receptors (CBR) [15]. It can be suggested that a certain role is also played by the peripheral benzodiazepine receptors (PBR), the number of which varies under stress conditions similarly to that of CBR [8]. PBRs participate in the regulation of synthesis of steroids and insulin and modify the level of adrenocorticotropic hormone [15]. However, the mechanisms of these effects are poorly understood. PBR are localized predominantly on the mitochondrial membranes. In association with potential-dependent anion channels and adenine nucleotide carriers they functionally integrate the internal and external mitochondrial membranes [14]. The ligands that specifically bind to PBR reduce the respiratory control in the mitochondria, change the activity of mitochondrial enzymes [7], and modify membrane structural parameters [14]. These data indicate that benzodiazepines may modulate cell energy metabolism via PBR both under normal and cold conditions. In addition, as membrane-targeted lipophilic drugs, they can participate in nonspecific dynamic interaction with membranes and induce both local and generalized modifications of the membrane lipid phase [14].

Our aim was to study with the help of fluorescent analysis the effects of diazepam on structural and functional parameters of the thymocyte membrane after exposure to cold: lipid viscosity, lipoprotein interactions, transmembrane potential ($\Delta\psi_T$ and sum of $\Delta\psi$ across the plasma and mitochondrial membranes), and the level of lipid peroxidation (LPO) products. The effect of benzodiazepines on LPO is a matter of controversy, the point being further complicated by the difference of the drug's effects *in vivo* and *in vitro* [1]. The thymocytes were chosen because they have a typical set of organelles and receptors for many

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hormones and transmitters, the benzodiazepines included [9].

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats weighing 190-240 g. The rats were kept on unrestricted food and water *ad libitum* diet in plastic cages (8-10 per a cage) at room temperature under controlled illumination with 1:1 light/darkness cycle. The control rats were injected with 0.5 ml physiological saline, the test rats were given diazepam (Relanium, Polfa) in a dose of 5 mg/kg dissolved in the same volume. After 1 h the rats were placed into cold (1-2°C) into motility-restraining plastic cages that had a large number of temperature regulating holes. The rats were cooled for a period necessary to decrease the rectal temperature to 3°C. Temperature was measured using a TPEM electronic thermometer with transducer inserted to the depth of about 6 cm. The rats were killed by decapitation. The thymocytes were isolated using the standard method [10]. After a single-pass washing in Hanks' saline without phenol red, the cells were resuspended in the same saline. Fluorescent analysis [3] was used to measure $\Delta\psi_T$ with the help of the fluorescent cation probe 4-(*n*-dimethylaminostyryl)-1-methylpyridinium (DSM, Zonde) and to determine the viscosity of membrane lipids with a pair of fluorescent probes: pyrene (Serva) and 4-(*n*-dimethylaminostyryl)-1-dodecylpyridinium (DSP-12, Zonde). We also compared the ratios of pyrene excimerization efficiency to radiation-free transfer from pyrene to DSP-12 in the control liposomes of known viscosity and in the studied membranes. Lipoprotein interactions in thymocyte membrane were evaluated by recording the resonance-induced energy transfer from tryptophan residues of membrane proteins to pyrene located in the lipid bilayer membrane.

The contents of the following LPO products were determined: conjugated dienes by absorption spectra of lipid extracts in ultraviolet light (the extracts were prepared with 0.001% ionol at 4°C) [4], and malonic dialdehyde (MDA) by the level of 2-thiobarbituric acid reactive substances. Fluorescence was measured at room temperature in an MPF-4 spectrofluorimeter (Hitachi) in a circular cuvette with diameter 0.5 cm and cell content 6×10^6 cell/ml. Hitachi-556 spectrophotometer was used for spectrophotometry.

RESULTS

Diazepam produced no statistically significant changes in the body temperature under the comfortable thermal conditions: 37.0±0.12°C in the control and 36.6±0.16°C in diazepam-treated rats. One hour postinjection there was a decrease in $\Delta\psi_T$ and 1.3-fold decrease in the membrane lipid viscosity ($p<0.001$, Table 1). However, thymocytes did not demonstrate any changes in the lipid-protein relations and in the level of LPO products.

To ascertain whether the revealed changes are related to the peripheral effects of diazepam, we studied its effects in cell suspension. Incubation of thymocytes of intact rats in Hanks' solution with diazepam (0.2 μg/ml, 37°C) for 15 min and injection of the drug *in vivo* decreased $\Delta\psi$. In addition, it increased lipid viscosity by 30% (Table 2). Incubation of thymocytes with the drug did not produce any significant changes in the intracellular content of LPO products (conjugated dienes and MDA Table 2), indicating that diazepam did not intensify oxidation. To evaluate its antioxidative properties, LPO induction is necessary. At the same time, these data indicate that diazepam-induced changes in $\Delta\psi$ and membrane lipid viscosity in thymocytes are not related to changes in LPO intensity.

Exposure of control rats to cold decreased $\Delta\psi$ and the level of conjugated dienes (Table 1). Similar changes occur in rat thymocytes during the first 2-5 h of adaptation to cold, according to the method of Hart. They are accompanied by compensatory activation of the antioxidant systems against the background decrease in viscosity of membrane lipids [5,10]. The effect of low temperatures on restrained rats increased the viscosity of membrane lipids in thymocytes (Table 1). Under free behavior within the framework of Hart's model its increase was observed only after a 24-h

TABLE 1. Effect of Diazepam on Structural and Functional Parameters of the Thymocyte Membrane in the Rats Exposed to Cold (*M*±*m*)

Index	Control (*n*=12)	Cold (*n*=10)	Diazepam (*n*=8)	Diazepam+cold (*n*=7)
$\Delta\psi_T$, mV	208±2.3	196±3.0**	193±2.8*	205±3.7**
Conjugated dienes, A_{233}/mg of lipids	0.92±0.02	0.69±0.07**	1.15±0.13	0.93±0.12*
Lipid viscosity, P	0.78±0.03	0.95±0.06**	0.60±0.03*	0.83±0.06**
Lipid-protein interaction, %	41±1.24	37±1.8	40±2.3	38±1.9

Note. $p<0.05$: *relative to control, **relative to warm conditions.

TABLE 2. Effect of Diazepam on the Thymocyte Membrane after Addition to Incubation Medium (*M*±*m*)

Index	Control	Diazepam
$\Delta\psi_T$, mV	212±5.2	198±2.2*
Conjugated dienes, A_{233}/mg of lipids	0.98±0.07	0.84±0.06
Lipid viscosity, P	0.81±0.02	1.12±0.08**
Lipid-protein interaction, %	41±1.7	39±2.1

Note. *$p<0.05$, **$p<0.01$ relative to control.

exposure to cold [10]. In diazepam-treated rats, cold exposure increased $\Delta\psi_T$ to the values characteristic of the control rats under comfortable thermal conditions. The viscosity of membrane lipids in the test rats also increased ($p<0.01$), although this parameter returned to its initial value that was decreased by diazepam. Cold exposure produced no statistically significant changes in the lipid-protein interactions and in the content of LPO products in the thymocytes of the test rats.

Under the effect of diazepam, the physicochemical parameters of the thymocyte membrane became similar to those in rats subjected to moderately low temperatures for 7 weeks [10]: $\Delta\psi_T$ decreased, which reflected uncoupling between oxidation and phosphorylation, and the viscosity of membrane lipids also decreased. In rats adapted to cold these structural and functional changes in the thymocyte membrane correlated with changes in other tissues and are considered as generally adaptive in nature [5]. In our experiments during the acute phase of cold exposure (when the specialized adaptive mechanisms are not yet fully activated) the temperature was maintained due to ATP consumption [4]. In this case diazepam contributes to the maintenance of the conjugation of oxidation and phosphorylation on the cold, but not under comfortable thermal conditions, as indicated by high $\Delta\psi_T$ level. This may also be assisted by prevention by diazepam of an extra increase in the membrane lipid viscosity induced by cold. Diazepam is known to prevent an increase in oxygen consumption under cold [11], which may be considered as an indication of a moderate cold-induced metabolic response. Presumably, in this case temperature is maintained due to activation of some mechanisms of thermal insulation (for example, vascular reaction). From our findings it can be concluded that changes in the physicochemical properties of cell membranes induced by interaction of diazepam with PBR may play a certain role in enhanced tolerance to cold produced by this drug.

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