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Characterization of Benzodiazepine Receptors on Human Lymphocytes

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It is demonstrated that benzodiazepine binding sites on human peripheral blood lymphocytes are the peripheral type receptors. The binding sites for the selective agonist ^{3}H-Ro 5-4864, but not for the antagonist ^{3}H-PK 11195, are completely inactivated by freezing-thawing of lymphocytes. The binding of ^{3}H-Ro 5-4864 to intact lymphocytes is activated by GABA and (+)baclofen. It is shown that the selective ligands Ro 5-4864 and PK 11195 bind to different subtypes of benzodiazepine receptors on human peripheral blood lymphocytes.

Key Words: *benzodiazepines; receptors; human lymphocytes*

The concept on benzodiazepine (BD) receptors was formulated after discovery of specific binding sites with high affinity for BD derivatives (diazepam) in the mammalian brain [1,7]. A correlation between the binding of BD preparations to these sites and their therapeutic effects has been established [2-4]. Benzodiazepines have been widely used in clinical practice as tranquilizers [2]. It was found that BD-receptors are located in the central nervous system (CNS), some organs and tissues, and on lymphocytes [6,8,9]. According to the affinity of selective ligands for BD-receptors, the receptors were divided into central and peripheral [5]. The BD-receptors located on lymphocytes are poorly investigated [8].

The BD derivatives diazepam and flunitrazepam bind both to central and peripheral receptors. The selective ligand Ro 15-1788 binds predominantly to the central BD-receptors. The agonist Ro 5-4864 and antagonist PK 11195 are selective ligands of peripheral BD-receptors [10,11]. In the CNS, BD-receptors are coupled to GABA-receptors; GABA and its functional analogs increase the binding of the agonists of central receptors and have no effect on their binding to peripheral receptors [13].

The receptors of human blood cells, particularly those of lymphocytes, which are the key component of the immune system, are of particular interest because the state of blood cell receptors may reflect the state of the CNS receptors. Our objective was to study the binding of various selective ligands to BD-receptors of human peripheral blood lymphocytes and to typify these receptors.

MATERIALS AND METHODS

The following reagents were used: sodium and potassium chloride, disodium phosphate and potassium dihydrophosphate (Fluka), lithium, calcium and magnesium chloride, EDTA (Merck), and Ficoll-Paque (Pharmacia), bovine serum albumin, Trypan Blue, Ro 15-1788, Ro 5-4864, PK 11195 (Hoffman La Roche), diazepam, GABA (Serva), and (+)baclofen and (-)baclofen (Hoffman La Roche).

Mononuclear leukocytes (lymphocytes) were isolated by the standard method with some modifications. Blood was collected from healthy male donors (age 20-35 years) in siliconized glass vials

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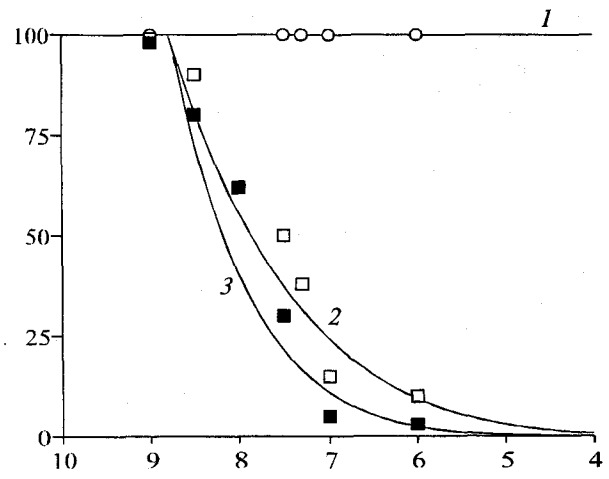

Fig. 1. Replacement of ^{3}H-flunitrazepam (9 nM) on human lymphocytes by Ro 5-4864 *(3)*, diazepam *(2)*, and Ro 15-1788 *(1)*. Abscissa: negative logarithm of the unlabeled ligand concentration; ordinate: residual specific binding, %.

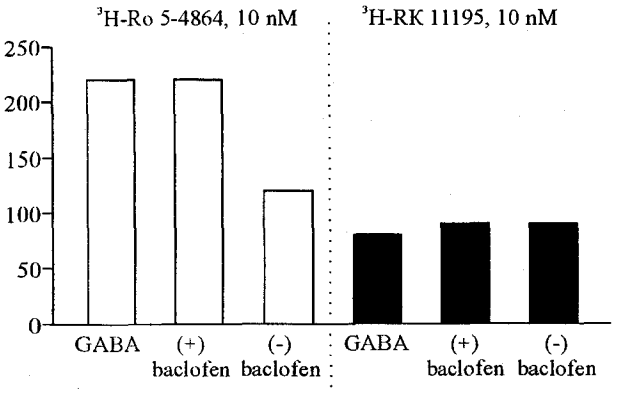

Fig. 2. Effects of GABAergic effectors on specific binding of peripheral ligands of the benzodiazepine receptors to intact lymphocytes (the maximum binding in the absence of an effector is taken as 100%).

with 0.38% sodium citrate. Erythrocytes were separated by centrifugation (10 min, 250*g*). Plasma enriched with leukocytes was layered on a Ficoll-Paque gradient (d=1.077 g/cm^3, 2-3 volumes plasma per one volume of Ficoll-Paque) and centrifuged for 40 min at 400*g*. The ring at the interface, which consisted of lymphocytes, was aspirated, and the cells were washed twice at 4°C with phosphate buffer containing (in mM): 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, and 1.5 KH_2PO_4, pH 7.4, (buffer I), centrifuged 15 min at 400*g*, and resuspended in buffer I to a final concentration of 10^6-10^7 cells/ml. Goryaev's chamber was used to assess the purity of resultant suspension and determine its cell content. The suspension contained >90% lymphocytes, >95% of which were viable according to the Trypan Blue exclusion test.

The membranes were prepared by lymphocyte homogenization in a Teflon-glass homogenizer with subsequent freezing in liquid nitrogen and thawing.

The binding of labeled ligands (NEN): ^{3}H-flunitrazepam, (85 Ci/mmol), ^{3}H-RO 5-4864 (86 Ci/mmol), and ^{3}H-PK 11195 (75 Ci/mmol) was measured after their addition to the lymphocyte suspension in buffer I. Nonspecific binding was determined in the presence of 10^{-6} M unlabeled ligands.

Samples were incubated at 4°C for 30 min. The binding was stopped by adding the excess of buffer I and subsequent vacuum filtering on Whatman GF-B filters pretreated with 0.1% polyethylenimine (Serva). Radioactivity was measured in a Mark-III scintillation counter with count efficiency 40%.

RESULTS

The competitive replacement method was used to typify the BD-receptors on blood lymphocytes. Radiolabeled flunitrazepam, which binds to both central and peripheral BD-receptors, served as a ligand. The specific antagonist of the central receptors Ro 15-1788, the selective ligand of peripheral receptors Ro 5-4864, and the ligand of both receptors diazepam were the replacing agents. Ro 15-1788 practically did not replace ^{3}H-flunitrazepam on lymphocytes (Fig. 1). The peripheral agonist Ro 5-4864 actively competed with ^{3}H-flunitrazepam, judging from IC_{50} equal to 90 nM. The replacing activity of diazepam toward ^{3}H-flunitrazepam was practically the same as that of Ro 5-4864: IC_{50}=120 nM.

These findings allow us to classify the lymphocyte BD-receptors as peripheral receptors.

It was interesting to characterize peripheral BD-receptors on human lymphocytes using selective ligands: the agonist ^{3}H-Ro 5-4864 and antagonist ^{3}H-PK 11195.

TABLE 1. Parameters of the Binding of Selective Ligands of Peripheral BD-Receptors to Human Lymphocytes

Preparation	Ligand			
	^{3}H-RO 5-4864		^{3}H-PK 11195	
	K_d, nM	B_{max}, fmol/mg protein	K_d, nM	B_{max}, fmol/mg protein
Intact lymphocytes	55	1400	16	2400
Lymphocyte membranes	No binding	No binding	10	3940

Scatchard plots of ^{3}H-PK 11195 binding to intact lymphocytes and their membranes has shown that this dependence is linear, pointing to one type of binding sites. The dissociation constants were similar for intact lymphocytes and their membranes: 16 and 10 nM, respectively. The maximum concentration of the binding sites (B_{max}) for ^{3}H-PK 11195 on membrane lymphocytes were 2400 and 3940 fmol/mg protein, respectively. Scatchard plots showed one type of binding sites for ^{3}H-Ro 5-4864 on human lymphocytes with K_d=55 nM and B_{max}= 1400 fmol/kg protein. The receptors for the selective ligand ^{3}H-Ro 5-4864 were not detected in the lymphocyte membrane preparations. These receptors are probably inactivated during freeing-thawing. The parameters of the selective ligand binding to peripheral BD-receptors on human lymphocytes are summarized in Table 1.

While comparing the parameters of the binding of peripheral ligands to lymphocyte BD-receptors, it can be hypothesized that ^{3}H-PK 11195 and ^{3}H-RO 5-4864 interact with two different subtypes of the receptors. To check up this hypothesis, we examined the effects of GABA and GABAergic ligands on the binding of ^{3}H-PK 11195 and ^{3}H-RO 5-4864 to the receptors on intact lymphocytes. Figure 2 shows the effect of GABA and the stereoselective GABAergic ligands (+)baclofen and (-)baclofen on the binding of these agents to lymphocytes. As the figure shows, the effects of GABAergic ligands are different. The addition of 5 mM GABA increases the binding of ^{3}H-Ro 5-4864 twofold and decreases that of ^{3}H-PK 11195 by 30%. The effects of baclofen stereoisomers on ^{3}H-PK 11195 binding were weak, while the binding of ^{3}H-Ro 5-4864 increased 2-fold under the effect of (+)baclofen in comparison with that of (-)baclofen.

It was hypothesized that GABA is involved in the regulation of peripheral BD-receptors *in vivo* [12]. Our experiments with GABA and baclofen show that there are two types of peripheral BD-receptors on human peripheral blood lymphocytes.

Thus, the binding of the selective ligand ^{3}H-Ro 5-4864 to human lymphocytes increases in the presence of GABA and (+)baclofen. In contrast to ^{3}H-PK 11195, ^{3}H-Ro 5-4864 does not bind to lymphocyte membranes prepared by homogenization and freezing-thawing. Human peripheral blood lymphocytes express the peripheral type BD-receptors; ^{3}H-RO 5-4864 and ^{3}H-PK 11195 bind to different subtypes of these receptors.

REFERENCES

1. A. V. Bogatyrskii, S.A. Andronati, and N. Ya. Golovenko, *Tranquilizers (1,4-Benzodiazepines) and Related Structures* [in Russian], Kiev, (1980).
2. A. Ya. Korneev, R. G. Mukhin, and I. I. Faktor, *Vestn. Akad. Med. Nauk. SSSR,* No. 1, 20-27 (1982).
3. V. A. Raiskii, *Psychotropic Drugs in Internal Diseases* [in Russian], Moscow (1988)
4. L. K. Ryago, R. K. Kiivet, and L. Ch. Allikmets, *Byull. Eksp. Biol. Med.,* **104,** No. 12, 685-687 (1987).
5. P. V. Sergeev and N. L. Shimanovskii, *Receptors* [in Russian], Moscow (1987).
6. D. Cahard, X. Canat, P. Karanon, *et al., Lab. Invest.*, **1**, 27-31 (1994).
7. E. Costa, P. Greengard, *Mechanism of Action of Benzodiazepines,* New York (1975).
8. C. Ferrarese, M. Perego, C. Marzorati, *et al., Neuropharmacology,* **34**, No. 4, 427-431 (1995).
9. C. Ferrarese, M. Perego, C. Marzorati, *et al., Int. J. Neurosci.*, **17**, No. 2, 141-145 (1996).
10. P. Ferrero, P. Rocca, A. Gualerzi, *et al. J. Neurol. Sci.*, **102**, No. 2, 209-219 (1991).
11. R. W. Olsen, *J. Neurochem.*, **37**, No. 1, 1-13 (1981).
12. R. F. Squires, in: *Benzodiazepine Receptors. Handbook of Neurochemistry,* London-New York (1984), pp. 261-306.
13. T. I. Taniguchi, J. K. T. Wang, and S. Spector, *Biochem. Pharmacol.*, **31**, No. 4, 589-590 (1982).