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CHARACTERIZATION OF PERIPHERAL-TYPE BENZODIAZEPINE BINDING SITES ON HUMAN LYMPHOCYTES AND LYMPHOMA CELL LINES AND THEIR ROLE IN CELL GROWTH

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Abstract—Peripheral-type benzodiazepine binding sites (PBRs) are ubiquitous in mammalian tissues. However, the physiological role of PBRs has not yet been clarified. In this study we characterized a saturable and high affinity binding site for [^{3}H]Pk 11195 (isoquinoline carboxamide derivative) on human lymphocytes and different lymphoma cell lines. Binding parameters of the human T-lymphoma cell line CCRF-CEM came closest to values for lymphocyte binding. Thus, the CCRF-CEM cell line appears to be a suitable lymphocyte cell model for further study of PBRs. To evaluate the pharmacological specificity of binding to human lymphocytes and CCRF-CEM cells we investigated the potency of different ligands to displace [^{3}H]Pk 11195 from its binding site. Pk 11195 was found to be the most potent inhibitor followed by 4′-chlorodiazepan (Ro5-4864) and diazepam (range of inhibition constants from 6.7×10^{-9} M to 3.6×10^{-7} M), whereas ligands specific for the central-type receptor like clonazepam and flumazenil had no displacing potency in the tested concentration range (10^{-10}–10^{-4} M). Since it was assumed that PBRs might be involved in the regulation of cell growth and differentiation, we studied the influence of PBR ligands on cell growth and survival using a quantitative colorimetric assay (MTT). Ligands which bind selectively to PBRs inhibited cell multiplication *in vitro*. However, half-effective concentrations (EC_{50}) were in the micromolar range and above therapeutic *in vivo* concentrations (range of EC_{50} values from 2.4×10^{-5} M to 1.5×10^{-4} M). Clonazepam and flumazenil had no inhibiting potency in the tested concentration range (10^{-10}–10^{-4} M). Although the difference between values for displacing potency and ability to inhibit cell multiplication cannot be explained as yet, it is interesting that all PBR-ligands followed the same sequence in displacing [^{3}H]Pk 11195 and inhibiting cell multiplication and that central type ligands were ineffective in both assays. This association suggest a mediating role of PBR binding in cell growth.

Benzodiazepines are among the most frequently prescribed drugs used primarily as anxiolytics, hypnotics and anticonvulsants. Their therapeutic effects are mediated through high affinity receptors in the central nervous system coupled to the GABA (γ-aminobutyric acid) anion channel receptor complex [1, 2]. At the same time a second binding site for benzodiazepines has been characterized in peripheral tissues [2] but its physiological role has not yet been clarified.

These so-called "peripheral-type" benzodiazepine binding sites (PBRs‡) are ubiquitous in mammalian tissues [3] such as the kidneys, the adrenals, the heart, the testes, blood cells and some neuronal regions [4, 5]. PBRs could be shown to be located in mitochondrial outer membranes [6] and hence exert influence on mitochondrial respiratory control [7]. Molecular characterization of the PBR binding protein identified a 17-kDa protein [8] with five potential hydrophobic transmembrane-spanning regions but it cannot be assigned to a special group of membrane proteins yet [9]. The ligands Ro5-4864 (4′-chlorodiazepan) and Pk 11195 (isoquinoline derivative) bind specifically to PBRs while having only low affinities to central-type benzodiazepine receptors. Specific ligands for the central receptor like clonazepam have negligible affinities to PBRs, whereas diazepam binds to central- and peripheral-type benzodiazepine receptors [10].

Influences of PBR-mediated actions on a variety of biological processes have been described such as release of Ca^{2+} from rat heart and kidney mitochondria [11], and stimulation of steroid synthesis in cell systems [12]. The number of PBRs is significantly increased in different brain tumors [13] and in the temporal cortex of patients with Alzheimer's disease [14] which suggests a possible role of PBRs as a marker for certain brain functions.

Diazepam has been described earlier as having immunosuppressive properties [15, 16]. Thus, after characterization of PBRs on different cells which take part in the immunoreaction, the influence of PBRs on immunological functions is discussed: PBR ligands stimulate monocyte chemotaxis *in vitro* [17] and exert immunomodulating activities by binding to macrophages [18]. Furthermore, it has been shown that benzodiazepine receptor ligands modulate T-cell function in antigen-specific systems [19]. In

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‡ Abbreviations: PBR, peripheral-type benzodiazepine binding site; HBSS, Hanks' buffered salt solution; PBS, phosphate-buffered saline.

this context, it should be mentioned that PBRs might be involved in the growth of and differentiation in other cells [20–22].

Therefore, we evaluated in this study the binding characteristics of [^{3}H]Pk 11195 to human lymphocytes and various human lymphoma cell lines which might provide suitable cell models for further studies of PBRs. Concomitantly, we investigated the displacement potency of different ligands to evaluate the pharmacological specificity of [^{3}H]Pk 11195 binding. In addition, cell growth experiments were performed to elicit a possible physiological role of PBR binding in these cells.

MATERIALS AND METHODS

Materials. Ligands were obtained from: Du Pont de Nemours (Dreieich, Germany)—[^{3}H]Pk 11195 ([^{3}H]1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide); Fluka (Buchs, Switzerland)—Ro5-4864; Hoffmann La Roche AG (Grenzach-Wyhlen, Germany)—diazepam, clonazepam and flumazenil. Pk 11195 was a gift from Rhône-Poulenc (Vitry sur Seine, France). Scintillation fluid Aquasafe 300 was from Zinsser Analytic (Frankfurt, Germany); RPMI 1640 medium (Cat. No. F1285), HBSS (Hanks' buffered salt solution), mitogens and Ficoll solution were from Biochrom KG (Berlin, Germany); CG medium was from Camon (Wiesbaden, Germany); and scrubbed nylon wool type 200L was from Du Pont de Nemours. Monoclonal antibodies were from Becton Dickinson (Mountain View, CA, U.S.A.).

Cell preparation. Peripheral mononuclear cells were isolated from heparinized venous blood of healthy volunteers (eight female and seven male, age range 25–32 years) using Ficoll density gradient centrifugation: 10 mL blood were diluted with an equal volume of HBSS pH 7.4 and layered carefully on 15 mL of Ficoll solution (density 1.077 g/mL). Centrifugation was performed at 500 *g* for 30 min at room temperature (20–22°). The interfacial layer containing the lymphocytes was transferred to a clean tube and washed twice with HBSS. Lysis of residual erythrocytes was accomplished with 0.83% NH_4Cl/PBS (phosphate-buffered saline) for 7 min at 37° and lymphocyte fraction was subsequently washed twice with HBSS. Monocytes were eliminated from T- and B-cells by adherence to tissue culture plates for at least 2 hr. The lymphocyte pellet was resuspended in medium (*ca.* 1×10^8 cells/2 mL) and transferred to a nylon wool column (10-mL disposable syringe was packed with 0.6 g scrubbed nylon wool and preincubated with RPMI 1640 medium for 1 hr at 37°). The T-cell fraction could be eluted with approximately 50 mL medium after incubation of the column for 1 hr at 37°. Monoclonal antibodies (CD 3, CD 19) were used to check the purity of the T-cell fractions using the Becton Dickinson procedure for indirect immunofluorescence staining of cell surfaces. CCRF-CEM cells and K 562 cells were kindly provided by Dr V. Gekeler, University Tübingen Department of Physiological Chemistry, Germany. Molt-4B cells were kindly provided by Prof. F. Mechelke, University Hohenheim Department of Genetics, Germany. Raji cells were bought from Flow Laboratories (Meckenheim, Germany). The cell lines were grown in CG medium and lymphocytes were grown in RPMI 1640 medium containing 10% fetal calf serum. Both media were supplemented with 100 U penicillin G sodium and 100 μg/mL streptomycine sulfate. All cells were cultured at 37° in a humidified atmosphere of 95% air/5% CO_2. Cell preparations were adjusted to 1×10^7 cells/mL in PBS pH 7.4 for binding studies.

Binding studies. Radioligand binding studies were carried out by incubation of 200 μL of the cell suspensions with [^{3}H]Pk 11195 at concentrations ranging from 1.2 to 19.2 nM at 4° for 40 min in a final volume of 500 μL PBS. (Kinetic experiments at 4° to determine the association and dissociation constants revealed achievement of binding equilibium within 10 to 15 min). The reaction was stopped by rapid filtration over Whatman GF/B filters including two washing steps with 5 mL ice-cold incubation buffer. Incubation vials were kept on ice during the procedure. Dry filters were counted in 5 mL Aquasafe 300 scintillation fluid using a Beckmann LS 1800 counter.

Specific binding ("bound") was defined as radioactivity bound in the absence of 10 μM diazepam minus non-specific binding which was evaluated using 10 μM diazepam as competing ligand. The data were analysed by Scatchard plots. For displacement studies, a stable concentration of the radioligand (1.2 nM [^{3}H]Pk 11195) was incubated with increasing concentrations (10^{-10}–10^{-4} M) of different unlabeled ligands dissolved in dimethyl sulphoxide. The data were analysed using logit-log plots. Protein content of different cells was measured according to the method of Bradford [23].

Cell multiplication assay. For growth experiments, human lymphocytes were stimulated with phytohemagglutinin 2.4 μg/mL in RPMI 1640 medium. CCRF-CEM cells were cultured in CG medium. Lymphocytes and CCRF-CEM cells (5×10^4 per well) were exposed to different ligand concentrations (10^{-10}–10^{-4} M, dissolved in ethanol 96%) for 4 and 3 days, respectively, in multiwell culture plates. Cell multiplication was measured using the quantitative colorimetric MTT assay as described previously [24]. After incubation of cells with a tetrazolium salt, mitochondrial dehydrogenases of viable cells build a purple formazan reaction product which can be measured with a spectrophotometer at a wavelength of 570 nm. The MTT assay shows results comparable with those estimated with the [^{3}H]thymidine incorporation assay [24], but has the advantage of omitting any radioactivity. Hill plot estimation was used to calculate concentrations which caused 50% inhibition of cell multiplication (EC_{50} values).

All presented data are corrected for the negligible effects of the solvents and were evaluated from three separate experiments. Each value represents the mean of triplicate determinations.

Protein binding. To evaluate the binding of diazepam (0.3–2.5 μM) to proteins in cell culture media, we analysed the diazepam concentration in both sides of a dialysis chamber after equilibrium dialysis (2 hr at 37°) using gas chromatography [25].

RESULTS

[^{3}H]*Pk* 11195 *binding to different cell populations*

Radioligand binding studies with [^{3}H]Pk 11195

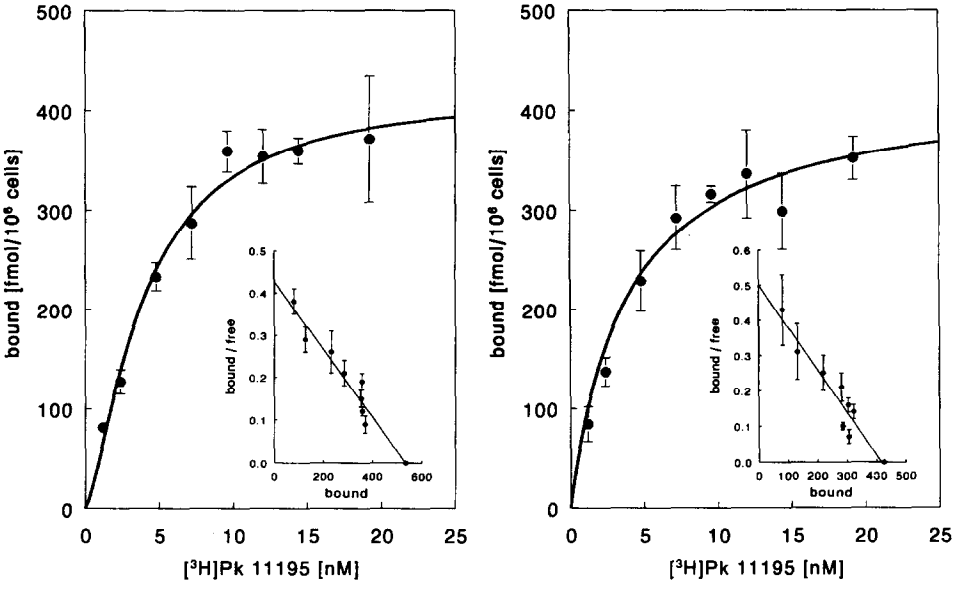

Fig. 1. (a) Specific binding of [^{3}H]Pk 11195 to human lymphocytes shown as saturation curve. Insert figure depicts the Scatchard plot of the same data. Each value represents mean ± SD of three separate experiments. (b) Specific binding of [^{3}H]Pk 11195 to CCRF-CEM cells shown as saturation curve. Insert figure depicts the Scatchard plot of the same data. Each value represents mean ± SD of three separate experiments.

Table 1. Characteristics of binding studies with different cell populations and [^{3}H]Pk 11195

Cell population	K_d (nM)	B_{max} (fmol/1 × 10^6 cells)	B_{max} (pmol/mg protein)
T- and B-lymphocytes	5.1 ± 1.2	535 ± 98	9.9 ± 1.8
T-lymphocytes	6.9 ± 0.9	522 ± 7	12.6 ± 0.2
CCRF-CEM (T-lymphoma cell line)	3.9 ± 1.7	425 ± 41	3.3 ± 0.3
Raji (B-lymphoma cell line)	1.8 ± 0.5	291 ± 4	1.6 ± 0.1
K 562 (myelogenous leukemia cell line)	2.0 ± 0.2	259 ± 19	1.0 ± 0.1
Molt-4B (T-lymphoma cell line)		No significant specific binding	

Values represent means ± SD, N = 3.

characterized a single, high affinity specific binding site on different cell populations (Fig. 1a and b). Specific binding was found to be a saturable process with no significant difference between the maximal binding capacities (B_{max}) of mixed T- and B-cell populations and pure T-cell fractions but distinctly lower B_{max} values for human lymphoma cell lines. Equilibrium dissociation constants (K_d) of [^{3}H]Pk 11195 binding were in the nanomolar concentration range and slightly lower for lymphoma cell populations. The human lymphoma cell line Molt-4B was the only cell population not suitable for radioligand binding studies with [^{3}H]Pk 11195. Values for binding capacity and affinity constants for the T-lymphoma cell line CCRF-CEM came closest to the binding parameters of lymphocytes (Table 1).

To evaluate the pharmacological specificity of binding to human lymphocytes and CCRF-CEM cells, we tested different ligands in a concentration range from 10^{-10} to 10^{-4} M in displacement studies with the radioligand [^{3}H]Pk 11195. The ligand's ability to displace [^{3}H]Pk 11195 from its binding sites in human lymphocytes corresponded to that of CCRF-CEM cells. Pk 11195 had the highest displacing potency followed by Ro5-4864 and diazepam, whereas ligands specific for the central-type benzodiazepine receptor like clonazepam and flumazenil had no displacing potency in the tested concentration range (Table 2).

Effects of PBR ligands on lymphocyte multiplication in vitro

After incubation of cells with different ligands we found Pk 11195, Ro5-4864 and diazepam to be inhibitors of cell multiplication *in vitro*. Pk 11195

Table 2. Results of displacement studies

Ligand	IC_{50} (nM) Human lymphocytes	IC_{50} (nM) CCRF-CEM cell line
Pk 11195	6.7 ± 1.5	14.9 ± 5.0
Ro5-4864	60.2 ± 1.4	95.3 ± 14.0
Diazepam	310.1 ± 191.6	365.0 ± 208.9
Clonazepam	No displacement up to 10^{-4} M	No displacement up to 10^{-4} M
Flumazenil	No displacement up to 10^{-4} M	No displacement up to 10^{-4} M

IC_{50} values represent half-maximal concentrations to inhibit [^{3}H]Pk 11195 binding.
Values represent means ± SD, N = 3.

Table 3. Results of growth experiments

Ligand	EC_{50} (μM) Human lymphocytes	EC_{50} (μM) CCRF-CEM cell line
Pk 11195	28.6 ± 3.8	24.5 ± 2.0
Ro5-4864	32.3 ± 16.5	36.0 ± 3.2
Diazepam	84.0 ± 14.5	146.4 ± 31.3
Clonazepam	No effect up to 100 μM	No effect up to 100 μM
Flumazenil	No effect up to 100 μM	No effect up to 100 μM

EC_{50} values represent concentrations which caused 50% inhibition of cell multiplication.
Values represent means ± SD, N = 3.

acted as the most potent inhibitor followed by Ro5-4864 and diazepam, whereas clonazepam and flumazenil had no inhibitory potency in the tested concentration range 10^{-10}–10^{-4} M (Table 3). Concentrations which reduced cell multiplication by 50% (EC_{50}) compared with control populations were at the micromolar level.

Investigation of diazepam binding to medium protein showed that the protein fraction of RPMI 1640 medium (10% fetal calf serum) bound approximately 50% and the protein fraction of CG medium (1% bovine serum albumin) bound about 20% of this ligand.

DISCUSSION

Specific benzodiazepine binding sites have been identified in human lymphocytes in previous studies [26, 27]. In this study, the binding characteristics of [^{3}H]Pk 11195 to human lymphocytes and different lymphoma cell lines, the affinity of various compounds to PBRs on these cells and the influence of different PBR ligands on cell growth were evaluated.

After determination of binding parameters for different cell lines we found results for CCRF-CEM cells comparable with those for lymphocytes. Thus, the CCRF-CEM cell line appears to be a suitable cell model for the further study of PBRs and their physiological role in lymphocytes (see Table 1). No significant difference between binding parameters for mixed T- and B-cell populations and pure T-cell fractions could be observed. The assumption that specific PBRs are present on both T-lymphocytes and B-lymphocytes was also supported by the observation that PBRs could be characterized on a T-lymphoma cell line (CCRF-CEM) and on a B-lymphoma cell line (Raji).

In competitive binding studies different ligands were able to displace [^{3}H]Pk 11195 from its binding site. Whereas specific ligands of central-type binding sites like clonazepam and flumazenil were ineffective, Pk 11195 and Ro5-4864 exhibited strong competitive activity. The displacement potency of the ligands followed the same order in human lymphocytes and CCRF-CEM cells: Pk 11195 > Ro5-4864 > diazepam (see Table 2).

Benzodiazepines have been described as modulating the differentiation of a number of cell populations which express PBRs such as glioma cells [21, 28] and thymoma cells [22]. In our study, we investigated a possible influence of PBR ligands on lymphocyte growth *in vitro*. Pk 11195, Ro5-4864 and diazepam significantly inhibited multiplication of PHA-stimulated lymphocytes and CCRF-CEM cells in the micromolar concentration range whereas clonazepam and flumazenil were again found to be ineffective (see Table 3). There was a qualitative correlation between cell multiplication and competitive binding assays: the ability of the ligands to

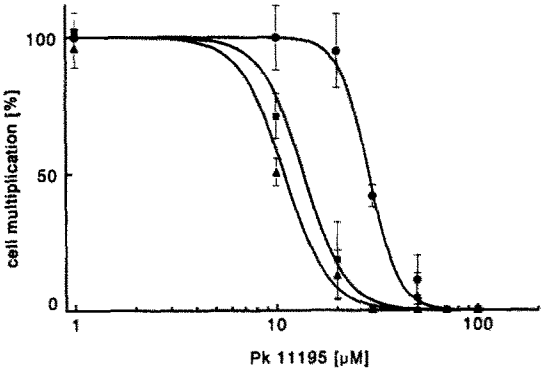

Fig. 2. Effect of Pk 11195 on CCRF-CEM cell multiplication with no addition (●), and after addition of 36 µM Ro5-4864 (▲) or 100 µM diazepam (■). Each value represents mean ± SD of three separate experiments.

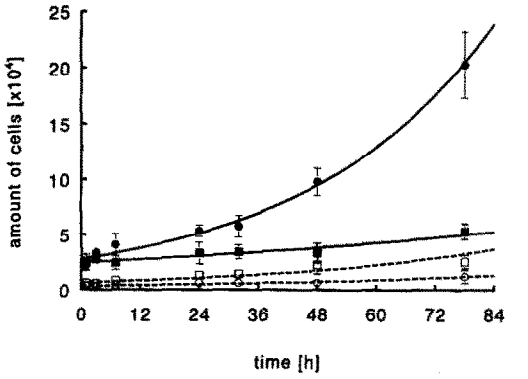

Fig. 3. Increase in dead CCRF-CEM cells (broken lines, open symbols) and living cells (solid lines, closed symbols) during incubation with 30 µM Pk 11195 (squares) compared with controls (circles). Each value represents mean ± SD of three separate experiments.

inhibit cell growth and their potency in displacement experiments with the cell populations tested followed the same order and clonazepam and flumazenil were inactive in both experiments. However, binding of Pk 11195 was nearly 10 times stronger than binding of Ro5-4864, but both ligands showed similar effects in growth experiments (see Tables 2 and 3). Further experiments indicated the inhibitory effect of different PBR ligands to be additive: a shift to the left of the Pk 11195 dose–respone curve was observed after the addition of half-effective concentrations of Ro5-4864 or diazepam (see Fig. 2), whereas clonazepam and flumazenil had no significant effect (data not shown). Generally, Ro5-4864 and Pk 11195 are considered as "agonist" and "antagonist" of PBRs, respectively [5]. However, in terms of cell growth both showed similar effects indicating that this biological process might be affected by any specifically bound ligand irrespective of its classification in other systems. Our observations are in accordance with other investigators who reported parallel effects of the two ligands on murine lymphocyte proliferation [29].

It remains to be discussed whether the inhibition of cell multiplication represents a toxic effect caused by high ligand concentrations or whether it is a non-specific effect caused by penetration of these lipophilic ligands through cell membranes [30]. A pure toxic effect seems unlikely because we observed only a slow increase in dead cells during the incubation period compared with control (see Fig. 3). The close association between displacement potency and inhibiting capability of the ligands would suggest a specific effect, especially considering that there is no significant difference in the lipophilicity of the ineffective ligands clonazepam and flumazenil compared with that of the effective ligands. As discussed by other authors, the difference in the ranges of IC_{50} and EC_{50} values might be due to the binding of PBR ligands to proteins added to the cell culture media [22]. This could reduce free concentrations of the ligands during proliferation in comparison with binding experiments which were performed in buffer. However, we found that binding of diazepam to proteins in cell culture media was not high enough to explain the discrepancy between radioligand binding parameters and effective concentrations to inhibit cell proliferation *in vitro*.

The striking difference between IC_{50} and EC_{50} values could also be due to two different peripheral binding sites with binding affinities at nano- and micromolar ligand concentrations as has been found for the central-type benzodiazepine receptor [31]. Furthermore, the PBRs with K_d values in the nanomolar concentration range seem to be associated with 18-kDa protein, but additional peripheral binding sites (associated with a 30-kDa protein fraction) have been identified [12, 32].

The relationship between the binding of ligands to PBRs and inhibition of lymphocyte multiplication by these PBR ligands suggests a relationship between PBR binding and the physiological functions of lymphocytes. This hypothesis is also supported by the observation that T-lymphocyte proliferation in rat offspring was inhibited after prenatal administration of Ro5-4864 [29]. However, further investigations are needed to elicit the possible interaction of PBRs with the immune system.

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