Biochemical Pharmacology, Vol. 35, No. 2, pp. 227–230, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

CHARACTERIZATION OF PERIPHERAL BENZODIAZEPINE BINDING SITES IN HUMAN TERM PLACENTA

FUAD FARES and MOSHE GAVISH*

Rappaport Family Institute for Research in the Medical Sciences and Department of Pharmacology, Faculty of Medicine, Technion-Israel Institute of Technology, P.O.B. 9697, Haifa 31096, Israel

(*Received* 26 *April* 1985; *accepted* 18 *July* 1985)

Abstract—Peripheral benzodiazepine binding sites were characterized in human term placental membranes using [^{3}H]PK 11195, which is a ligand specific for peripheral benzodiazepine binding sites. Binding of [^{3}H]PK 11195 to human term placental membranes was found to be saturable. Scatchard analysis revealed a single population of binding sites ($r = 0.98$). Equilibrium dissociation constant (K_D) was 2.1 ± 0.3 nM, and density of binding sites (B_{max}) was 920 ± 105 fmol/mg protein. The K_D value calculated from kinetic experiments was 3.6 ± 0.2 nM. The ability of various drugs to displace [^{3}H]PK 11195 from human term placental binding sites was tested: the inhibition constants (K_I) for PK 11195, Ro 5-4864, and diazepam were 2.9, 11.8, and 177 nM, respectively, whereas clonazepam, methyl-β-carboline-3-carboxylate, Ro 15-1788, chlordiazepoxide, atropine, and estradiol were inefficient in displacing [^{3}H]PK 11195 ($K_I > 10^{-5}$ M).

High-affinity binding sites for benzodiazepines (BZs)† have been demonstrated in the central nervous system [1, 2]. The binding of various BZs to these sites correlates with their clinical potency as anticonvulsants and anxiolytics [3]. Pharmacological studies suggest that BZs exert their therapeutic effects by facilitating synaptic actions of the major inhibitory neurotransmitter in the CNS, γ-aminobutyric acid [4–6].

In addition to the "central" BZ receptors located in the CNS, another type of BZ binding sites, called "peripheral" sites, have been described, initially in peripheral tissues [7–10], but also in the brain [11–14]. These binding sites are different from central BZ receptors in their distribution within the brain, their lack of coupling to the GABA receptors, and their specificity for ligand binding. Clonazepam, which has high affinity for the central type, has very low affinity for peripheral sites, whereas the reverse is true with regard to Ro 5-4864. The isoquinoline carboxamide derivative [^{3}H]PK 11195 has been shown to bind with high affinity to human and rat platelets [15] and to rat brain cortex [16].

Behavioral and electrophysiological studies have shown that the ligand Ro 5-4864 increases sensitivity to audiogenic seizures in mice and that PK 11195 antagonizes this effect [17]. The convulsant activity of Ro 5-4864 has been attributed to an interaction with the *t*-butylbicyclophosphorothionate (TBPS) site [18]. PK 11195, which has been found to possess both convulsant and anticonvulsant properties [19], has also been found to antagonize the decrease in intracellular action-potential duration induced by Ro 5-4864 in guinea-pig heart preparation [20].

Peripheral BZ binding sites have been demonstrated in endocrine glands such as adrenal [21] and hypophysis [22]. BZs induce reduction of the release of peptide hormones from the rat pituitary such as prolactin [23] and ACTH [24]. In the study reported here we investigated the possible existence of BZ-binding sites on human term placenta, which is a highly active endocrine organ during pregnancy. It is a major source of steroids [25] and of protein hormones [26]. Demonstrating the existence of high-affinity peripheral BZ-binding sites in the membranes of human term placenta, as reported in this paper, prepares the ground for future research on the effects of BZ treatment on peripheral BZ-binding sites in human term placenta and on the release of hormones from this organ.

MATERIALS AND METHODS

Materials. [^{3}H]PK 11195 was purchased from New England Nuclear (Boston, MA). Unlabeled BZs were kindly supplied by Drs H. Gutmann and E. Kyburz (Hoffman-La Roche, Basel, Switzerland). Unlabeled PK 11195 was a generous gift from Dr G. Le Fur (Pharmuka Laboratories, Gennevilliers, France). All other compounds were purchased from commercial sources.

Membrane preparation. One placenta was obtained immediately after elective cesarean section at normal human pregnancy. Sections of villous tissue were immediately washed several times with ice-cold Tris–HCl buffer (50 mM, pH 7.4) and frozen at −20°. Preparation of membranes for binding studies was as previously described [27]. The tissue (1 g) was defrosted and homogenized in 50 vol. of Tris–HCl buffer at 4° with a Brinkman polytron (setting 10) for 15 sec. The homogenate was centrifuged at 49,000 *g* for 15 min, and the pellet was suspended in 50 vol. of 50 mM Tris–HCl buffer (pH 7.4) and used for binding studies.

Binding assay. Binding activity of peripheral BZ

* To whom all correspondence should be addressed.

† Abbreviations: BZ, benzodiazepine; K_D, equilibrium dissociation constant; k_{+1}, association rate constant; k_{-1}, dissociation rate constant.

sites was assayed in 50 mM Tris–HCl buffer, pH 7.4, in a final volume of 500 μl containing 400 μl placental membranes (200–300 μg protein) and 50 μl of [^{3}H]-PK 11195 (0.25–16 nM final concentration) in the absence (total binding) or presence (nonspecific binding) of 10 μM unlabeled Ro 5-4864. After incubation for 60 min at 4°, samples were filtered under vacuum over Whatman GF/B filters and washed three times with 5 ml of 50 mM Tris–HCl buffer, pH 7.4. Filters were placed in vials and counted for radioactivity.

RESULTS

Specific [^{3}H]PK 11195 binding at 4° in human term placental membrane was determined as a function of protein concentration. Specific binding was increased linearly in relation to protein concentration up to 0.5 mg per assay (Fig. 1). In subsequent experiments, we used 0.2–0.3 mg protein per assay.

Concentration dependence of specific binding of [^{3}H]PK 11195 at equilibrium is shown in Fig. 2. Specific binding of [^{3}H]PK 11195 reached a plateau at 8 nM. Nonspecific binding was measured in the presence of 1 μM of unlabeled Ro 5-4864. Nonspecific binding at 5 nM of [^{3}H]PK 11195, a concentration used routinely in our binding studies, was about 25% of total binding. Scatchard analysis of saturation curves of [^{3}H]PK 11195 binding to human term placental membranes yielded a linear Scatchard plot ($r = 0.98$), which indicated the presence of a single population of binding sites. The equilibrium dissociation constant (K_D) was 2.1 nM, and the maximal binding capacity (B_{max}) was 920 fmol/mg protein (Fig. 2).

The kinetics of [^{3}H]PK 11195 binding to human term placental membranes is presented in Fig. 3. Specific binding at 4° attained equilibrium at 45 min, with half-maximal binding at 7 min. The plot ln $B_{eq}/(B_{eq} - B)$ vs time had a slope (K_{ob}) of 0.117 min^{-1}. The rate constant for association of [^{3}H]PK 11195 binding to peripheral BZ sites in human term placenta calculated from these experiments and from the dissociation constant rate (see below) was $k_{+1} = 0.0136$/min per nM.

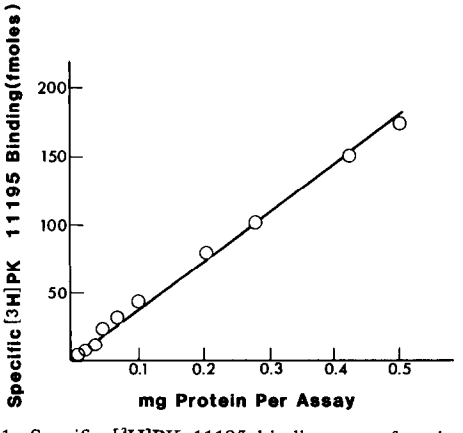

Fig. 1. Specific [^{3}H]PK 11195 binding as a function of protein concentration. Aliquots of human term placental membrane ranging from 0.008 to 0.5 mg protein were incubated with 2 nM [^{3}H]PK 11195 for 60 min at 4°, alone or in the presence of 1 μM unlabeled Ro 5-4864. The points represent the mean value of three experiments in one placenta with less than 15% variability.

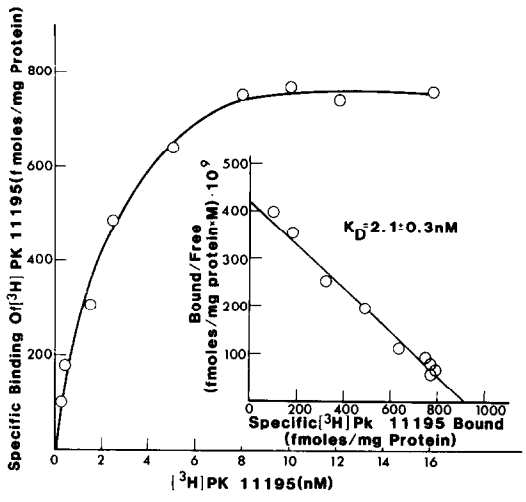

Fig. 2. Saturation curve of specific [^{3}H]PK 11195 binding to human term placental membrane. Membranes (0.3 mg protein) were incubated with different concentrations of [^{3}H]PK 11195 (final concentration 0.2–16 nM) for 60 min at 4°. Inset shows Scatchard plot of [^{3}H]PK 11195 binding to human term placental membrane. The line is a regression line ($r = 0.98$). Values shown are the means of three separate experiments in one placenta with less than 15% variability.

The dissociation rate constant (k_{-1}) of [^{3}H]PK 11195 was determined by measuring bound [^{3}H]PK 11195 at various times after adding 1 μM of unlabeled Ro 5-4864 (final concentration) to incubation media of 5 nM [^{3}H]PK 11195 and human term placental membranes in which binding had attained equilibrium (Fig. 4). The dissociation of [^{3}H]PK 11195 was according to first-order kinetics, with half-life at 4° of 13.5 min and $k_{-1} = 0.049$ min^{-1}. The K_D value calculated from k_{+1} and k_{-1} was 3.6 nM.

In order to examine the pharmacological specificity of [^{3}H]PK 11195 binding sites in human term placenta, we tested the ability of various compounds to inhibit [^{3}H]PK 11195 specific binding (Table 1). This binding was not affected by 10 μM of flurazepam, chlordiazepoxide, methyl-β carboline-3-carboxylate, Ro 15-1788, clonazepam, atropine, or estradiol. Of all the drugs tested, PK 11195 was the most potent. Ro 5-4864 was about 4 times less potent than PK 11195, but about 15 times more potent than diazepam.

We also tested the stability of the peripheral BZ binding sites from human term placenta. After 1 hr at 4°, no loss of activity was observed; but after 1 hr at 25°, 37°, and 60°, loss of activity of 25%, 47%, and 75%, respectively, was observed.

DISCUSSION

In this study we demonstrated the existence of peripheral BZ binding sites in human term placental membranes by the isoquinoline carboxamide derivative [^{3}H]PK 11195, which is a ligand specific for peripheral BZ binding [15].

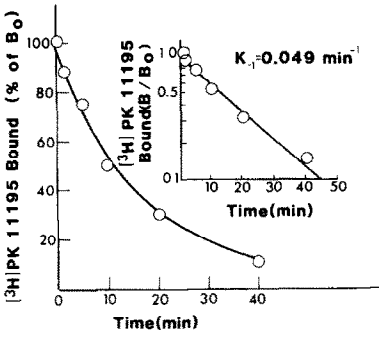

Fig. 3. Time course of specific [^{3}H]PK 11195 binding to human term placental membrane. Specific binding of [^{3}H]-PK 11195 (final concentration 5 nM) was determined as described under Materials and Methods. Inset shows calculation of the observed association constant (K_{ob}) from the equation $\ln B_{eq}/(B_{eq} - B_t) = K_{ob} \times t$, where B_{eq} and B_t are the concentrations of receptors at equilibrium and at time t, respectively. The association constant rate (k_{+1}) is calculated from the equation $k_{+1} = K_{ob}/L_t$, where L_t is the free ligand concentration. Specific binding was determined as described under Materials and Methods. Each point is the mean of three separate experiments in one placenta with less than 15% variability.

Binding of [^{3}H]PK 11195 to these sites is saturable, and Scatchard analysis revealed a single population of binding sites ($r = 0.98$). The K_D value from equilibrium binding experiments reported in other studies for [^{3}H]PK 11195 binding to peripheral BZ binding sites ranged from 0.87 nM in the rat cortex [16] to 6.43 nM in human platelet membranes [15].

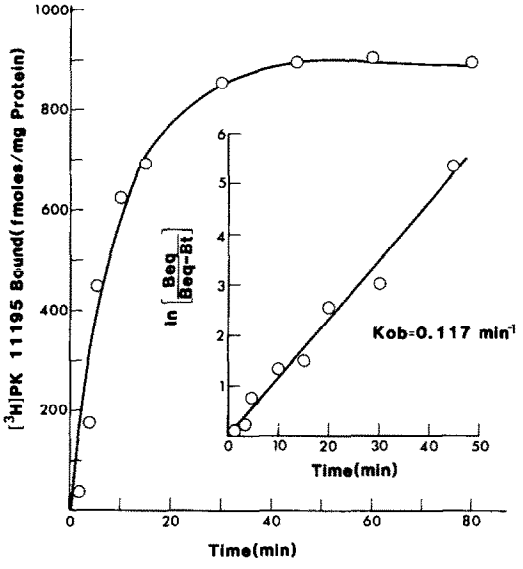

Fig. 4. Dissociation of bound [^{3}H]PK 11195 from human term placental membrane. Samples were incubated to equilibrium at 4° in the presence of 5 nM [^{3}H]PK 11195. Dissociation was begun by adding 1 μM unlabeled Ro 5-4864 (final concentration), and the samples were filtered immediately (zero time) and at times indicated. Specific binding was determined as described under Materials and Methods. Each point is the mean of three separate experiments in one placenta with less than 15% variability. [^{3}H]PK 11195 at t_0 was 650 fmol/mg protein.

Table 1. Inhibition of [^{3}H]PK 11195 binding to human term placental membranes by various compounds

Compound	K_i (nM)
PK 11195	2.9
Ro 5-4864	11.8
Diazepam	177
Flurazepam	>10,000
Chlordiazepoxide	>10,000
Methyl-β-carboline-3-carboxylate	>10,000
Ro 15-1788	>10,000
Clonazepam	>10,000
Atropine	>10,000
Estradiol	>10,000

Specific binding of [^{3}H]PK 11195 (final concentration 5 nM) to human term placental membranes was determined in the presence of 7–9 concentrations of several compounds in triplicate to estimate IC_{50} values (concentration causing 50% inhibition of [^{3}H]PK 11195 binding). The K_i values were calculated from the equation $K_i = IC_{50}/(1 + C/K_D)$, where C = [^{3}H]PK 11195 concentration and K_D = 2.1 nM. Results are the mean of three separate experiments in one patient with less than 15% variability.

The K_D value obtained in this study (2.1 nM) for peripheral BZ binding sites in human term placental membranes was similar to the values obtained in olfactory-bulb membranes [16], in rat cardiac membranes [28], and in rat platelets [15].

The B_{max} value reported in other studies on peripheral BZ-binding sites ranged from 128 fmol/mg protein in rat cortex [16] to 30,620 fmol/mg protein in rat platelet membranes [15]. The B_{max} value observed here for human term placental membranes reached 920 fmol/mg protein, which is 7 times higher than the value obtained for rat cortex [16], but 2–3 times lower than the value obtained for olfactory-bulb membranes and rat cardiac membranes [16, 28] and 33 times lower than the value for rat platelets [15].

The K_D value calculated from kinetic experiments was slightly higher than that calculated from equilibrium experiments (Figs. 3 and 4).

The potency of the variety of drugs tested to displace bound [^{3}H]PK 11195 from human term placental peripheral BZ binding sites (Table 1) was similar to that obtained in other peripheral BZ binding sites: PK 11195 was the most potent, while clonazepam, which binds to the central BZ receptors, demonstrated very low affinity to peripheral BZ binding sites [7–10].

The fact that treatment at 60° destroyed most peripheral BZ binding activity in human term placental membranes indicates the presence of a protein-binding site.

The placenta in later pregnancy is a major source of progesterone and estrogens, which play an important role in maintaining pregnancy [29]. Since BZs inhibit the release of prolactin from the hypophysis [23], the possible influence of BZs on the secretion of placental hormones should be investigated. Studies on the effect of BZs on the release of hormones are in progress on tissue-culture cells from human term placenta, which were also found to contain peripheral BZ-binding sites (data not shown).

Acknowledgements—This work was supported by the Fund for the Promotion of Research at the Technion. We thank Ruth Singer for typing this manuscript.

REFERENCES

1. R. Squires and C. Braestrup, *Nature, Lond.* **266**, 732 (1977).
2. H. Möhler and T. Okada, *Science* **198**, 849 (1977).
3. H. Möhler, T. Okada, Ph. Heitz and J. Ulrich, *Life Sci.* **22**, 985 (1978).
4. E. Costa and A. Guidotti, *A. Rev. Pharmac. Toxicol.* **19**, 531 (1979).
5. W. Haefely, A. Kulcsar, H. Möhler, L. Pieri, P. Polc and R. Schaffner, *Adv. biochem. Pharmac.* **14**, 131 (1975).
6. R. MacDonald and J. L. Barker, *Nature, Lond.* **271**, 563 (1978).
7. C. Braestrup and R. F. Squires, *Proc. Matl. Acad. Sci. U.S.A.* **74**, 3805 (1977).
8. T. Taniguchi, J. K. T. Wang and S. Spector, *Biochem. Pharmac.* **31**, 589 (1982).
9. T. Taniguchi, J. K. T. Wang and S. Spector, *Life Sci.* **27**, 171 (1980).
10. J. K. T. Wang, T. Taniguchi and S. Spector, *Life Sci.* **27**, 1881 (1980).
11. J. K. T. Wang, T. Taniguchi, M. Sugiura and S. Spector, *Pharmacologist* **23**, 160 (1981).
12. J. P. Marangos, J. Patel, J. P. Boulenger and R. Clarke-Rosenberg, *Molec. Pharmac.* **22**, 26 (1982).
13. H. Schoemaker, M. Bliss and H. I. Yamamura, *Eur. J. Pharmac.* **71**, 173 (1981).
14. R. R. H. Anholt, K. M. M. Murphy, G. E. Mack and S. H. Snyder, *J. Neurosci.* **4**, 593 (1984).
15. J. Benavides, D. Quarteronet, P.-F. Plouin, F. Imbault, T. Phan, A. Uzan, C. Renault, M.-C. Dubroeucq, C. Gueremy and G. Le Fur, *Biochem. Pharmac.* **33**, 2467 (1984).
16. J. Benavides, D. Quarteronet, F. Imbault, C. Malgouris, A. Uzan, C. Renault, M. C. Dubroeucq, C. Gueremy and G. Le Fur, *J. Neurochem.* **41**, 1744 (1983).
17. J. Benavides, F. Guilloux, D. E. Allam, A. Uzan, J. Mizoule, C. Renault, M. C. Dubroeucq, C. Gueremy and G. Le Fur, *Life Sci.* **34**, 2613 (1984).
18. B. A. Weissman, J. Cott, J. A. Jackson, G. T. Bolger, K. H. Weber, W. D. Horst, S. M. Paul and P. Skolnick, *J. Neurochem.* **44**, 1494 (1985).
19. S. E. File, *Br. J. Pharmac.* **83**, 471 (1984).
20. M. Mestre, T. Carriot, C. Belin, A. Uzan, C. Renault, M. C. Dubroeucq, C. Gueremy and G. Le Fur, *Life Sci.* **35**, 953 (1984).
21. G. Le Fur, M. L. Perrier, N. Vaucher, F. Imbault, A. Flamier, J. Benavides, A. Uzan, C. Renault, M. C. Dubroeucq and C. Gueremy, *Life Sci.* **32**, 1839 (1983).
22. M. M. Voigt, L. G. Davis and J. H. Wyche, *J. Neurochem.* **43**, 1106 (1984).
23. G. Schettini, M. J. Cronin, S. B. O'dell and M. MacLeod, *Brain Res.* **291**, 343 (1984).
24. G. Bruni, P. Dal-Pray, M. T. Dotti and G. Segri, *Pharmac. res. Commun.* **12**, 163 (1980).
25. H. Helling, D. Gattereau, Y. Lefebvre and E. Bolte, *J. clin. Endocr. Metab.* **30**, 624 (1970).
26. G. P. Hennen, *Archs int. Physiol. Biochem.* **73**, 689 (1965).
27. M. Gavish and F. Fares, *Eur. J. Pharmac.* **107**, 283 (1985).
28. G. Le Fur, N. Vaucher, M. L. Perrier, A. Flamier, J. Benavides, C. Renault, M. C. Dubroeucq, C. Gueremy and A. Uzan, *Life Sci.* **33**, 449 (1983).
29. A. P. Flint, in *A Continuing Controversy: Human Parturition* (Eds. M. Keirse, A. Anderson and J. Gravenhorst), p. 85. Martinus Nijhoff, Amsterdam (1979).