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Characterization of binding sites for the ω_3 receptor ligands [^{3}H]PK11195 and [^{3}H]RO5-4864 in human brain

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Abstract

The kinetics and pharmacology of the isoquinoline and benzodiazepine binding sites of the ω_3 or peripheral-type benzodiazepine receptors were studied using the specific ligands [^{3}H] 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one ([^{3}H]PK11195) and [^{3}H]1-(2-Chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide ([^{3}H]RO5-4864), respectively. Binding of both ligands was saturable, reversible, displayed nanomolar affinity, and best fit to a single site model. Occipital cortex and cerebellum displayed highest and lowest densities of binding sites respectively; for both ligands. B_{max} values of [^{3}H]PK11195 were several-fold higher than that of [^{3}H]RO5-4864 in all regions studied consistent with their binding to distinct subunits of the human peripheral-type benzodiazepine receptor heteromeric complex. However, the isoquinoline and benzodiazepine ligands were found to be mutually competitive at nanomolar concentrations suggesting allosteric interactions between these two sites. Competition binding experiments showed that the binding of both ligands was displaced by diazepam with K_i values in the nM range, and by clonazepam in the μM range. The novel peripheral-type benzodiazepine receptor ligand 2-(4-fluorophenyl)-*N,N*-di-*n*-hexyl-1H-indole-3-acetamide ($FGIN_{1-27}$) displaced only [^{3}H]PK11195 binding with high potency. Heterogeneity of the two sites is observed, manifested by their differential susceptibility towards detergents and alcohols. Histidine residue modification by diethylpyrocarbonate treatment abolished only [^{3}H]PK11195 binding but had no effect on [^{3}H]RO5-4864 binding. These studies demonstrate that the isoquinoline and benzodiazepine sites on the peripheral-type benzodiazepine receptor in human brain manifest many pharmacological characteristics that are distinct from each other and from rodent brain peripheral-type benzodiazepine receptors. © 1997 Elsevier Science B.V.

Keywords: Benzodiazepine receptor, peripheral-type; [^{3}H]RO5-4864; [^{3}H]PK11195; Brain, human

1. Introduction

Due to their anticonvulsant, anxiolytic, hypnotic and muscle relaxant properties, benzodiazepines are amongst the most commonly prescribed drugs. Benzodiazepines bind to two pharmacologically and anatomically distinct classes of binding sites (Braestrup and Nielsen, 1980). The binding site associated with the ionotropic $GABA_A$ receptor (Barnard et al., 1987) known as the 'central-type' benzodiazepine receptor (Möhler and Okada, 1977) is neuronal, localized on the plasma membrane. On the other hand, the so-called ω_3 or 'peripheral-type' benzodiazepine receptor is not associated with the $GABA_A$ receptor complex (Gavish et al., 1992) and is predominantly astrocytic in localization (Itzhak et al., 1993). Peripheral-type benzodiazepine receptors are localized mainly on the outer mitochondrial membrane in both the central nervous system (CNS) and peripheral tissues (Anholt et al., 1986; Antkiewicz-Michaluk et al., 1988). The peripheral-type benzodiazepine receptor exists as a hetero-oligomeric complex with distinct binding domains for benzodiazepines and isoquinoline carboxamides (Doble et al., 1985; Sprengel et al., 1989; Blahos II et al., 1995). Although the exact function of these receptors is not yet fully established, based on the observations of its association with the voltage-dependent anion channel and adenine nucleotide carrier with which it forms a pore mediating the translocation of cholesterol across the mitochondrial membrane to the site of cytochrome P-450, peripheral-type benzodiazepine receptors appear to be rate-limiting in steroidogenesis (Bernassau et al., 1993; Garnier et al., 1994).

Evidence suggests that peripheral-type benzodiazepine receptors play a role in hepatic encephalopathy (Lavoie et

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al., 1990; Giguère et al., 1992; Rao et al., 1994; Desjardins et al., 1997), epilepsy (Ducis et al., 1990), ischemia (Earley et al., 1996), thiamine-deficiency encephalopathy (Leong et al., 1994), regulation of cell growth and differentiation (Wang et al., 1984) as well as stress and anxiety (Gavish et al., 1992). [^{11}C]-PK11195 is useful in visualizing human gliomas (Junck et al., 1989) and quantitating ischemic brain lesions by positron emission tomography (Benavides et al., 1990; Cremer et al., 1992).

Several studies previously reported species- and organ-selective differences in the pharmacological properties of peripheral-type benzodiazepine receptors (Benavides et al., 1983; Cymerman et al., 1986; Awad and Gavish, 1987; Eshleman and Murray, 1989). Although peripheral-type benzodiazepine receptors have been well characterized in brain and the peripheral tissues of various rodent species (see review by Gavish et al., 1992) as well as in cultured astrocytes (Itzhak et al., 1993), studies on the pharmacological characteristics of the peripheral-type benzodiazepine receptor in human brain are sparse.

The present study was undertaken in order to further characterize the benzodiazepine (labelled by [^{3}H]RO5-4864) and isoquinoline (labelled by [^{3}H]PK11195) sites of the human brain peripheral-type benzodiazepine receptor as well as the comparative regional distribution of these sites in human brain.

2. Materials and methods

2.1. Materials

[^{3}H]PK11195 (specific activity, 86.9 Ci/mmol) and [^{3}H]RO5-4864 (specific activity, 86.5 Ci/mmol) were purchased from NEN (Mississauga, Ont.). Nonradioactive RO5-4864 was purchased from Fluka fine chemicals (St. Louis, MO). Pregnenolone sulfate was purchased from Research Biochemicals International (Natick, MA). All other chemicals were purchased from Sigma-Aldrich Canada (Mississauga, Ont.).

2.2. Brain samples

Postmortem human brain samples were obtained at autopsy from six subjects (5 male and 1 female) who were free from neurological, psychiatric or metabolic disorders at the time of death. In no case had patients been exposed to drugs known to affect either GABA or benzodiazepine receptors in the 30 days prior to death. Mean age ($\pm$SEM) for these subjects was 59 ($\pm$1.8) years (range, 52–65 years); mean postmortem delay time ($\pm$SEM) was 11.7 ($\pm$2.5) h (range, 7–20 h). Authorization from next to kin was obtained in all cases. Brains were dissected according to a standardized procedure developed in our laboratory and based on the atlas of De Armond et al. (1976). Immediately after dissection, individual regions were manually chopped and aliquoted (400–600 mg wet weight of tissue per vial) to ensure homogeneity in sampling as well as to limit each sample to one freeze–thaw cycle. Samples were frozen at -80°C until time of assay.

2.3. Radioligand binding assays

[^{3}H]PK11195 and [^{3}H]RO5-4864 binding assays were performed by the method of Rao et al. (1993). Briefly, thawed brain tissue was homogenized in 20 vol of ice-cold 50 mM Tris–HCl buffer (pH 7.4). The homogenate was centrifuged at 40,000 g for 20 min at 4°C, and the pellet was washed twice by rehomogenization in fresh buffer and repeated centrifugation (40,000 g for 20 min at 4°C). The final pellet was suspended in fresh buffer and frozen at -80°C until the day of assay. On the day of the assay, the membrane preparations were thawed and washed twice with ice-cold buffer (40,000 g for 20 min at 4°C). The final pellet was suspended in 10 vol of fresh buffer and used in the binding assays. Protein content of the membrane preparations was measured by the method of Lowry et al. (1951).

In all binding assays, incubations were initiated by the addition of the membrane preparation (60–70 μg of protein equivalent) to a final volume of 250 μl of 50 mM Tris–HCl buffer (pH 7.4) containing either 2 nM [^{3}H]PK11195 or 2 nM [^{3}H]RO5-4864 (except in saturation experiments where a concentration range of 0.25–36 nM was used). After incubating at 4°C for 2 h (except in the kinetic experiments, which required a range of incubation intervals), the reaction was terminated by the addition of ice-cold 50 mM Tris–HCl buffer followed by vacuum filtration through GF/B glass microfiber filters (pretreated with 0.3% polyethylenimine) using a Brandel cell harvester. The filters were washed rapidly twice with 5 ml of ice-cold buffer, dried, and radioactivity was determined by liquid scintillation spectrometry. Nonspecific binding was determined by inclusion of 2 μM PK11195 or 20 μM RO5-4864 respectively. Total and nonspecific binding was measured in parallel sets of tubes (in duplicate), and specific binding was defined as the difference between the two.

For the study of association, 60–70 μg protein equivalent of membranes was incubated with either 2 nM [^{3}H]PK11195 or 2 nM [^{3}H]RO5-4864 in 50 mM Tris–HCl buffer (pH 7.4) for increasing intervals (1–300 min) in the presence (nonspecific binding) or absence (total binding) of either 2 μM PK11195 or 20 μM RO5-4864. As association of both ligands reached a steady state within 90 min and as the specific binding was stable for at least 4 h at 4°C, the initial incubation time of 120 min was selected for the study of dissociation. Dissociation was examined by addition of excess unlabeled PK11195 (2 μM) or RO5-4864 (20 μM) to tubes containing membrane preparation and [^{3}H]PK11195 (2 nM) or [^{3}H]RO5-4864 (2 nM), followed by increasing periods of incubation (0.5–120 min).

Displacement studies were performed in the presence of varying concentrations of PK11195 (10^{-12} to 2×10^{-3} M), RO5-4864 (10^{-12} to 2×10^{-3} M), 2-(4-Fluorophenyl)-*N*, *N*-di-*n*-hexyl-1H-indole-3-acetamide ($FGIN_{1-27}$)(10^{-12} to 2×10^{-3} M), diazepam (10^{-12} to 2×10^{-3} M) and clonazepam (10^{-9} to 2×10^{-3} M). The effect of alcohols (ethanol and isopropanol) on [^{3}H]PK11195 (2 nM) or [^{3}H]RO5-4864 (2 nM) binding was studied at four concentrations (0.5%, 1%, 2% and 4%). All other compounds were tested at three concentrations (50 μM, 250 μM and 500 μM), unless otherwise specified in the figure legends.

For the study of the effect of detergents (Triton X-100, Tween-20 and deoxycholic acid) on [^{3}H]PK11195 or [^{3}H]RO5-4864 binding, cortical membranes were incubated for 2 h in 50 mM Tris–HCl buffer (pH 7.4) containing either 2 nM [^{3}H]PK11195 or 2 nM [^{3}H]RO5-4864 and varying concentrations of detergent (0.001% to 1%).

2.4. Histidine residue modification studies

Histidine residue modification of the peripheral-type benzodiazepine receptor was performed by the method of Eshleman and Murray (1989). Washed membrane preparations were suspended in 50 mM Tris–HCl buffer (pH 6.0) and incubated for 5 min with various concentrations of diethylpyrocarbonate (100–3000 μM). Reaction was terminated by the addition of 10 vol of 50 mM Tris–HCl buffer (pH 7.4) and centrifugation (30,000 g for 20 min). The pellet was resuspended in fresh buffer for use in binding assays.

2.5. Data analysis

Data from saturation experiments were analyzed using an iterative curve-fitting procedure to a single rectangular hyperbola (Rao and Butterworth, 1996). Scatchard analysis was performed by first-order nonlinear regression analysis of the data from saturation isotherms, and the binding parameters (K_d and B_{max}) were calculated from these plots. Competition binding data (displacement studies) were analyzed and the K_i values were calculated by the EBDA programme within RADLIG. Statistical analysis of the data was performed by analysis of variance (ANOVA) followed by the post hoc Dunnett multiple comparisons test.

3. Results

Initial experiments showed that the specific binding of both [^{3}H]PK11195 and [^{3}H]RO5-4864 to human cortical membranes increased linearly with increasing protein concentration (up to at least 200 μg). Binding of both ligands was optimal between pH 5 and 8. Varying buffer strength from 25 mM to 200 mM had no significant effect on the binding of either ligand (results not shown).

3.1. Saturation binding experiments

Specific binding of both [^{3}H]PK11195 and [^{3}H]RO5-4864 was saturable (Fig. 1A and B), and analysis of the corresponding Scatchard plots (Fig. 1A and B, insets)

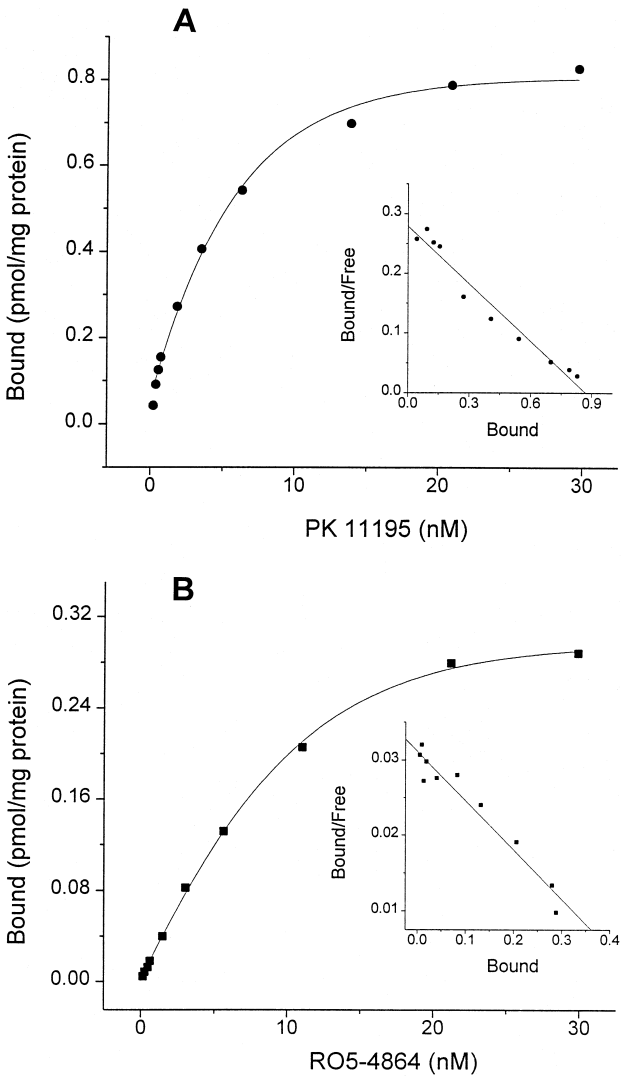

Fig. 1. Saturation isotherms and their corresponding Scatchard plots (insets) of specific [^{3}H]PK11195 binding (top panel) and [^{3}H]RO5-4864 binding (bottom panel). Specific binding is defined as the difference between total binding and nonspecific binding in the presence of either 2 μM PK11195 or 20 μM RO5-4864. Nonspecific binding was in all cases $<10\%$ for [^{3}H]PK11195 binding and $<30\%$ for [^{3}H]RO5-4864 binding at all ligand concentrations. The plots shown were from a representative experiment. Data are mean values of triplicate determinations. Incubations were for 2 h at 4^0C. 65–75 μg protein equivalent of membrane preparation from human occipital cortex was used in a total reaction volume of 250 μl. The [^{3}H]PK11195 or [^{3}H]RO5-4864 concentrations used were 0.25–36 nM. Scatchard analyses were performed using least square linear regression analysis. The mean $\pm$ SD ($n = 6$) B_{max} and K_d values are presented in Table 1.

showed a single class of high-affinity binding sites with no evidence of cooperativity, given the better fit to one-site rather than a two-site model and the near-unity Hill coefficients in the three human brain regions (occipital cortex, temporal cortex and cerebellum) studied. For both ligands the maximum binding site density (B_{max}) values were 2–3

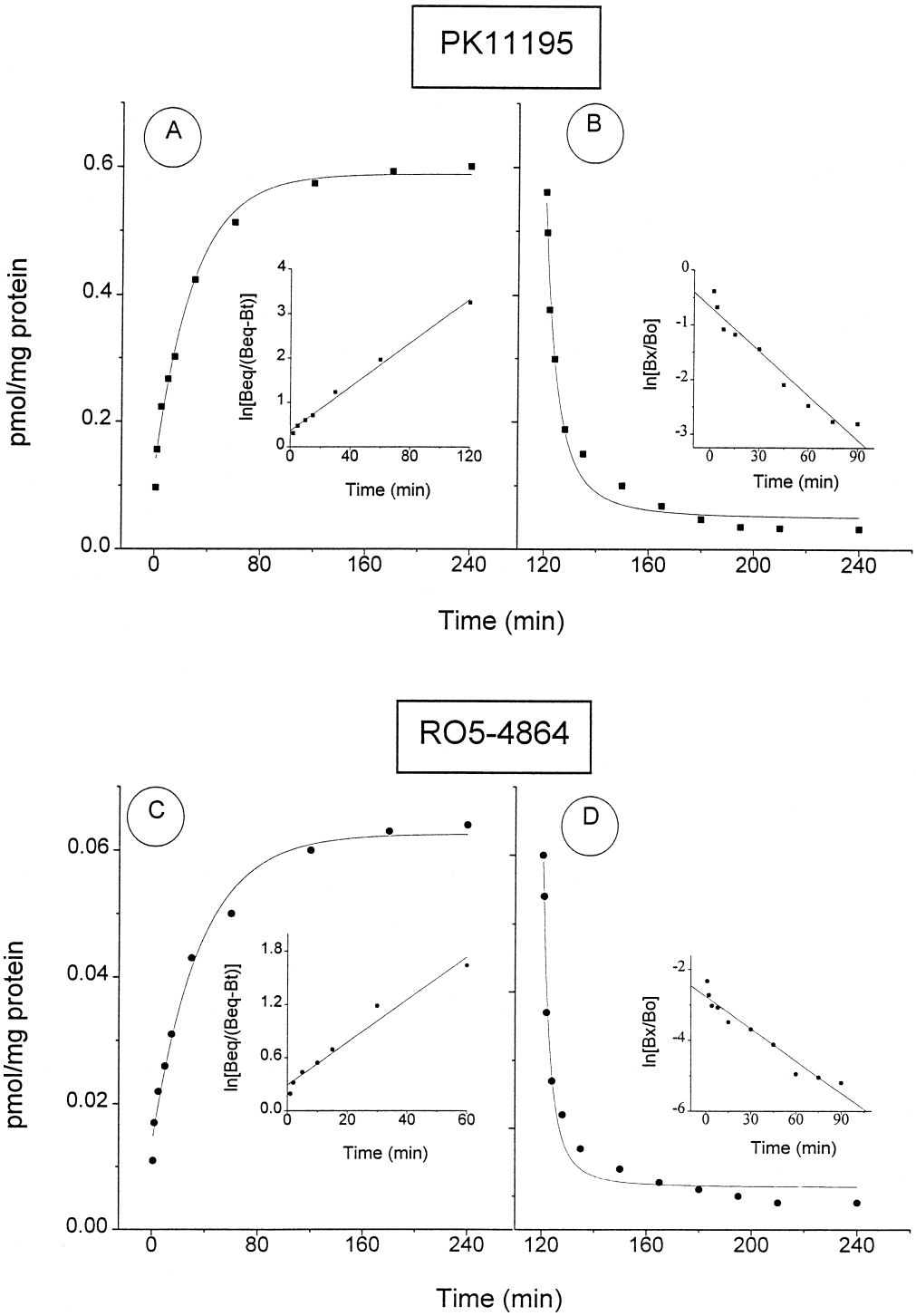

Fig. 2. Association (A and C) and dissociation (B and D) of specific [^{3}H]PK11195 binding (top panels) or [^{3}H]RO5-4864 binding (bottom panels) to human occipital cortical membranes. For measurements of association, 65–75 μg protein equivalent of membranes were incubated at 4°C for increasing intervals in the presence of 2 nM [^{3}H]PK11195 or [^{3}H]RO5-4864 in 50 mM Tris–HCl buffer (pH 7.4). Nonspecific binding was measured in the presence of either 2 μM PK11195 or 20 μM RO5-4864. For measurements of dissociation, membranes were incubated at 4°C in the presence of 2 nM [^{3}H]PK11195 or [^{3}H]RO5-4864 in 50 mM Tris–HCl buffer (pH 7.4) for 120 min to attain equilibrium. Specific binding was measured at increasing intervals after addition of either 2 μM PK11195 or 20 μM RO5-4864. In each case data points represent means of triplicate determinations of a representative experiment.

Table 1
Maximal densities and affinities of isoquinoline ([^{3}H]PK11195) and benzodiazepine ([^{3}H]RO5-4864) binding sites in human brain regions

Brain regions	[^{3}H]PK 11195		[^{3}H]RO5-4864	
	B_{max}	K_d	B_{max}	K_d
Occipital cortex	923 ± 69	2.65 ± 0.45	343 ± 77	13.17 ± 2.48
Temporal cortex	711 ± 58	2.88 ± 0.51	289 ± 91	16.66 ± 3.11
Cerebellum	346 ± 51	3.02 ± 0.49	136 ± 37	15.51 ± 3.45

Data are mean ± SD values of triplicate determinations in autopsied brain tissue from 6 patients. Parameters were determined by Scatchard analysis of saturation experiments (range of 0.25–36 nM of [^{3}H]PK11195 or [^{3}H]RO5-4864 and unlabelled PK11195 (2 μM) or RO5-4864 (20 μM) to define nonspecific binding). B_{max}, maximum binding site density; K_d, equilibrium dissociation constant. Units: B_{max} in fmol/mg protein and K_d in nM. Hill coefficients (n_H; cooperativity) were 0.98 to 1.04 range in all regions for both ligands.

times higher in cerebral cortex (occipital and temporal) than cerebellum (Table 1). In all regions, B_{max} values were ~3× higher for [^{3}H]PK11195 than [^{3}H]RO5-4864; whereas K_d values were ~5–6 times higher for [^{3}H]RO5-4864 than [^{3}H]PK111195 (Table 1). Neither B_{max} values nor K_d values showed any correlation with either age or postmortem delay time (data not shown).

3.2. Kinetic experiments

The association of [^{3}H]PK11195 and [^{3}H]RO5-4864 studied using cortical membranes (at 4°C) was observed to be monophasic and reached a steady state within ~1.5 h (Fig. 2A and C). The mean ± SD ($n = 3$) of the K_{obs} values calculated from the individual pseudo-first order plots of the association experiments were 0.026 ± 0.003 min^{-1} (for [^{3}H]PK11195 binding) and 0.027 ± 0.004 min^{-1} (for [^{3}H] RO5-4864 binding). Specific binding of both ligands was stable for at least 4 h. The dissociation studied after adding excess of unlabelled ligands for both [^{3}H]PK11195 and [^{3}H]RO5-4864 was rapid (Fig. 2B and D). In both cases, within 10 min, 80% of the bound ligand was dissociated. The mean ± SD ($n = 3$) of the dissociation rate constants calculated from the individual semilogarithmic plots of the dissociation experiments were 0.020 ± 0.002 min^{-1} ([^{3}H]PK11195 binding) and 0.024 ± 0.003 min^{-1} ([^{3}H]RO5-4864 binding). The ratio of the dissociation and association rate constants permitted estimation of K_d values of 6.6 nM (for [^{3}H]PK111195 binding) and 16 nM (for [^{3}H]RO5-4864 binding). These values are in good general agreement with K_d values determined by Scatchard analysis (2–3 nM for [^{3}H]PK111195 binding) and 12–17 nM (for [^{3}H]Ro5-4864 binding).

3.3. Regional distribution

Regional distribution of [^{3}H]PK11195 and [^{3}H]RO5-4864 binding sites studied at a ligand concentration of 2 nM in different human brain structures are presented in Table 2. In all ten brain structures studied, the binding of [^{3}H]PK11195 was several times higher than the binding of [^{3}H]RO5-4864. Highest densities of binding sites for both ligands were observed in the occipital cortex. Cerebellum showed the lowest density of binding sites for both ligands. Occipital cortex, frontal cortex, temporal cortex and the hippocampus manifested binding site densities in the 300–400 fmol/mg protein for [^{3}H]PK11195 and 35–48 fmol/mg protein for [^{3}H]RO5-4864. Thalamus and substantia nigra contained 200–300 fmol/mg protein of [^{3}H]PK11195 binding sites and 25–34 fmol/mg protein of [^{3}H]RO5-4864 binding sites. The binding of [^{3}H]PK11195 and [^{3}H]RO5-4864 were in the range of 100–200 fmol/mg protein and 15–24 fmol/mg protein, respectively in the globus pallidus/putamen, pons, caudate nucleus and cerebellum respectively (Table 2). The regional distribution of [^{3}H]PK11195 and [^{3}H]RO5-4864 binding sites were positively correlated (Fig. 3).

3.4. Displacement studies

Peripheral-type benzodiazepine receptors were shown to have two distinct binding sites which binds to PK11195 and RO5-4864 (Awad and Gavish, 1987). To characterize these two isoquinoline-carboxamide and benzodiazepine binding sites, various compounds were tested for their ability to displace [^{3}H]PK11195 (2 nM) and [^{3}H]RO5-4864 (2 nM) binding to cerebral cortical membranes. Fig. 4 shows the dose-response curves for these compounds. The mean ± SD ($n = 5$) of the K_i values obtained for various compounds are shown in Table 3.

Specific binding of both ligands studied was displaced by PK11195, RO5-4864 and diazepam with K_i values in the nM range. However, PK11195 was ~100 times more potent than RO5-4864 in displacing [^{3}H]PK11195 (2 nM) binding. On the other hand, RO5-4864 was only ~4 times

Table 2
Comparative regional distribution of isoquinoline ([^{3}H]PK11195) and benzodiazepine ([^{3}H]RO5-4864) binding sites on the peripheral-type benzodiazepine receptor in human brain

Region	[^{3}H]PK11195	[^{3}H]RO5-4864
Occipital cortex	377 ± 72	47 ± 11
Frontal cortex	322 ± 67	38 ± 7
Temporal cortex	306 ± 57	42 ± 9
Hippocampus	317 ± 89	44 ± 10
Cerebellum	101 ± 12	15 ± 4
Thalamus	274 ± 44	31 ± 8
Caudate nucleus	172 ± 36	24 ± 6
Pons	160 ± 39	25 ± 6
Globus pallidus/Putamen	117 ± 20	16 ± 3
Substantia nigra	235 ± 51	34 ± 8

Values are mean ± SD of specific binding determined in triplicate in brain homogenates from 6 different patients. Membrane preparations (100 μg) were incubated with either 2 nM of [^{3}H]PK11195 or 2 nM of [^{3}H]RO5-4864 in 50 mM Tris–HCl buffer (pH 7.4) for 2 h at 4°C. Nonspecific binding was determined in parallel incubations in the presence of either unlabelled PK11195 (2 μM) or RO5-4864 (20 μM).

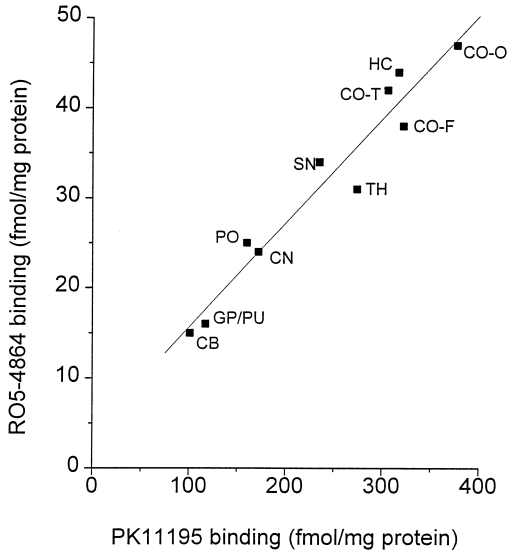

Fig. 3. Regional correlation between [^{3}H]PK11195 binding and [^{3}H]RO5-4864 binding site densities (B_{max}) in human brain. Each point represents the mean of specific binding determined in triplicate from 6 different subjects. In all cases, nonspecific binding (defined by using 2 μM PK11195 or 20 μM RO5-4864) was subtracted from the total binding studied using 2 nM [^{3}H]PK11195 or [^{3}H]RO5-4864 and 100 μg protein equivalent of membranes in a total volume of 250 μl of 50 mM Tris–HCl buffer (pH 7.4) for 2 h at 4°C. CO–O: occipital cortex; CO–F: frontal cortex; HC = hippocampus; CO–T = temporal cortex; TH = thalamus; SN = substantia nigra; PO = pons; CN = caudate nucleus; GF/PU = globus pallidus/putamen; CB = cerebellum.

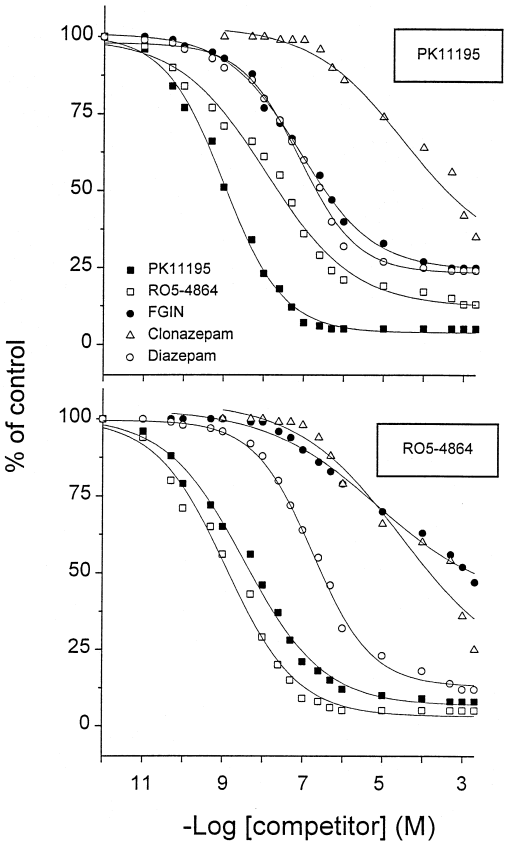

Fig. 4. Inhibition of [^{3}H]PK11195 binding (top panel) or [^{3}H]RO5-4864 binding (bottom panel) to human occipital cortical membranes by increasing concentrations (1×10^{-3} to 2×10^{6} nM) of various isoquinolines and benzodiazepines. Binding was studied using 2 nM [^{3}H]PK11195 or [^{3}H]RO5-4864 and 65–75 μg protein equivalent in a reaction volume of 250 μl. Incubations were for 120 min at 4°C in 50 mM Tris–HCl buffer (pH 7.4). Control values (100%) are 377 ± 47 ([^{3}H]PK11195 binding) and 40 ± 7 fmol/mg protein ([^{3}H]RO5-4864 binding). Data are from a representative experiment. Mean $\pm$ SD of K_i values calculated from 5 separate experiments are presented in Table 3.

more potent than PK11195 in displacing [^{3}H]RO5-4864 binding (Table 3). $FGIN_{1-27}$ displaced only [^{3}H]PK11195 binding with K_i values of 53 nM. Diazepam was three times more potent in displacing [^{3}H]RO5-4864 binding than [^{3}H]PK11195 binding (Table 3).

Clonazepam displaced the binding of [^{3}H]PK11195 and [^{3}H]RO5-4864 with less potency than diazepam, but was more potent (3 to 4 times) in displacing [^{3}H]RO5-4864 than [^{3}H]PK11195 (Table 3). The neurosteroids, pregnenolone and pregnenolone sulfate showed very low affinity for both the peripheral-type benzodiazepine receptor sites with high K_i values (> 100,000 nm) (Table 3). Other steroids including estradiol, progesterone and testosterone tested up to a concentration of 100 μM had no statistically significant effect on either [^{3}H]PK11195 or [^{3}H]RO5-4864 binding. Similarly, neurotransmitters (dopamine, norepinephrine, serotonin, GABA, glutamate and aspartate), aromatic amino acids (tryptophan and phenylalanine), branched-chain amino acids (valine, leucine and isoleucine), basic amino acids (histidine and arginine), sulfur containing amino acids (cysteine and methionine) and glutamine tested up to a concentration of 100 μM had no statistically significant effect on either [^{3}H]PK11195 or [^{3}H]RO5-4864 binding (data not shown).

3.5. Effect of dithiothreitol and detergents

Preincubation of human cortical membranes with the thiol reagent dithiothreitol (1 to 1000 μM) for 10 min, abolished the binding of both [^{3}H]PK11195 and [^{3}H]RO5-4864 in a dose-dependent manner with a maximal inhibition of 45–55% at 500 μM dithiothreitol (Fig. 5).

Table 3
Effects of various benzodiazepine receptor ligands and neurosteroids on [^{3}H]PK11195 and [^{3}H]RO5-4864 binding to human occipital cortex (K_i in nM)

	[^{3}H]PK11195	[^{3}H]RO5-4864
PK11195	0.303 ± 0.031	5.24 ± 1.43
RO5-4864	27.43 ± 8.92	1.45 ± 0.60
$FGIN_{1-27}$	52.99 ± 5.33	> 1000
Diazepam	42.42 ± 13.49	22.88 ± 7.9
Clonazepam	> 100,000	33,000
Pregnenolone	> 100,000	> 100,000
Preg. sulfate	> 100,000	> 100,000

Values represent mean $\pm$ SEM of 5 separate experiments. Binding of [^{3}HPK11195 (2 nM) and [^{3}H]RO5-4864 (2 nM) was studied by incubating 60–70 μg protein equivalent of membranes in 50 mM Tris–HCl buffer (pH 7.4) for 2 h at 4°C, in the presence of increasing concentrations (1×10^{-3} to 2×10^{6} nM) of the compound under examination.

Triton X-100, deoxycholic acid and Tween-20 had differential effects on the two peripheral-type benzodiazepine receptor sites (Fig. 5). [^{3}H]PK11195 binding was more resistant to the action of detergents than was [^{3}H]RO5-4864 binding. Triton X-100 at a concentration of 0.005% had no significant effect on [^{3}H]PK11195 binding but reduced [^{3}H]RO5-4864 binding by ~40%. At a concentration of 0.1%, Triton-X 100 inhibited [^{3}H]PK11195 binding by ~50% and [^{3}H]RO5-4864 binding by up to 85% (Fig. 5). Similarly, deoxycholic acid (0.1%) had no significant effect on [^{3}H]PK11195 binding but inhibited [^{3}H]RO5-4864 binding by ~50% (Fig. 5). At a concentration of 0.2%, deoxycholic acid inhibited [^{3}H]RO5-4864 binding by ~90% but decreased [^{3}H]PK11195 binding by only 30–35% (Fig. 4). Tween-20 (0.001–0.2%) inhibited [^{3}H]RO5-4864 binding in a dose-dependent manner with ~50% loss of binding at 0.04% concentration of the detergent (Fig. 5). On the other hand, [^{3}H]PK11195 binding was resistant to Tween-20 up to 0.2% concentration of detergent (Fig. 5).

3.6. Effect of alcohols

Both ethanol and isopropanol at concentrations of 2% and 4% significantly inhibited [^{3}H]RO5-4864 binding (Fig. 6). However, at both concentrations tested, isopropanol was more potent than ethanol. Isopropanol inhibited [^{3}H]RO5-4864 binding by 35% ($p < 0.05$) at concentrations as low as 0.5% (Fig. 6B). Neither of the alcohols tested (up to a concentration of 4%) had any significant effect on [^{3}H]PK11195 binding (Fig. 6A and B).

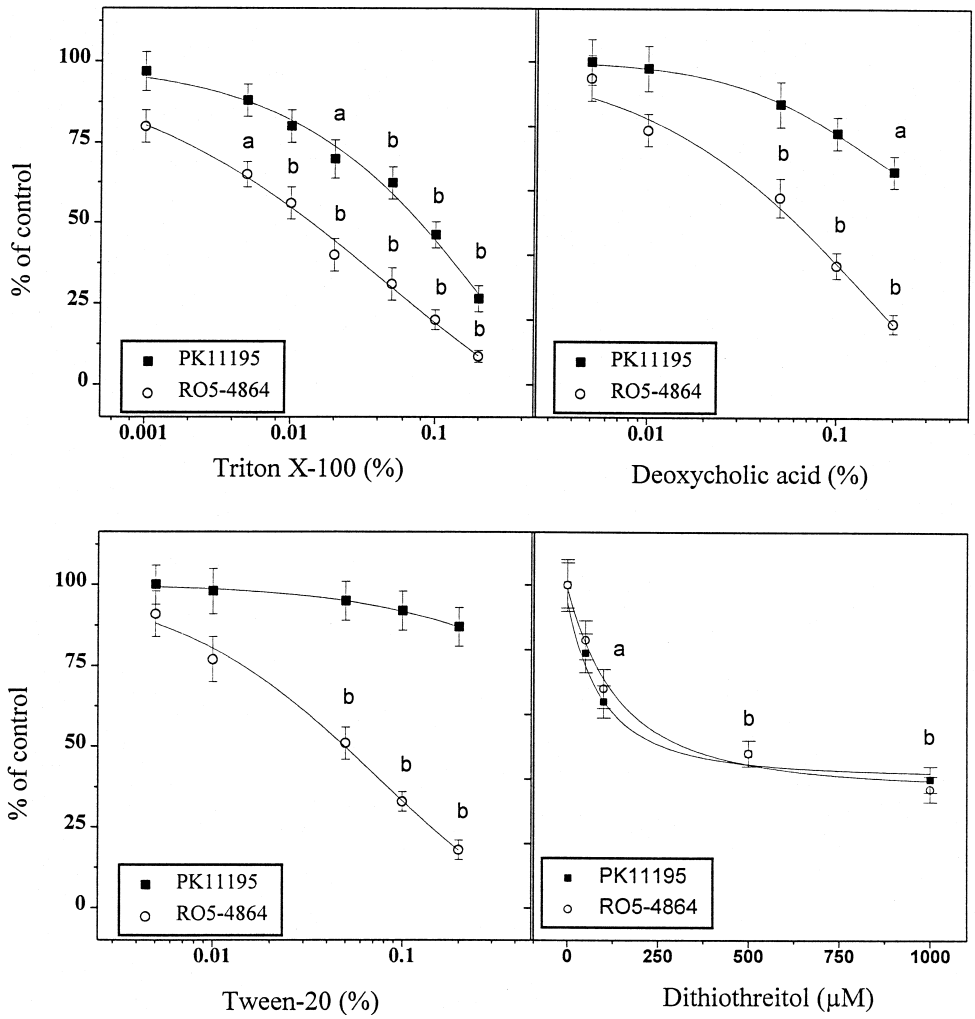

Fig. 5. Effect of various detergents (Triton X-100, deoxycholic acid and Tween-20) and dithiothreitol on [^{3}H]PK11195 and [^{3}H]RO5-4864 specific binding to human occipital cortical membranes. Values are given as percent control values. Control values (100%) are 361 ± 59 ([^{3}H]PK11195 binding) and 47 ± 11 fmol/mg protein ([^{3}H]RO5-4864 binding). Values are mean ± SD of 4 separate experiments. 65–75 μg protein equivalent of membranes were incubated for 120 min at 4°C in 50 mM Tris–HCl buffer (pH 7.4) containing 2 nM of either [^{3}H]PK11195 or [^{3}H]RO5-4864. Nonspecific binding was defined in the presence of either 2 μM PK11195 or 20 μM RO5-4864. The effects of detergents were studied at a concentration range of 0.001–1% and the effect of dithiothreitol was studied at a concentration range of 25 μM to 1000 μM. Statistics: $^aP < 0.05$ and $^bP < 0.01$ by ANOVA followed by Dunnett's multiple comparisons test.

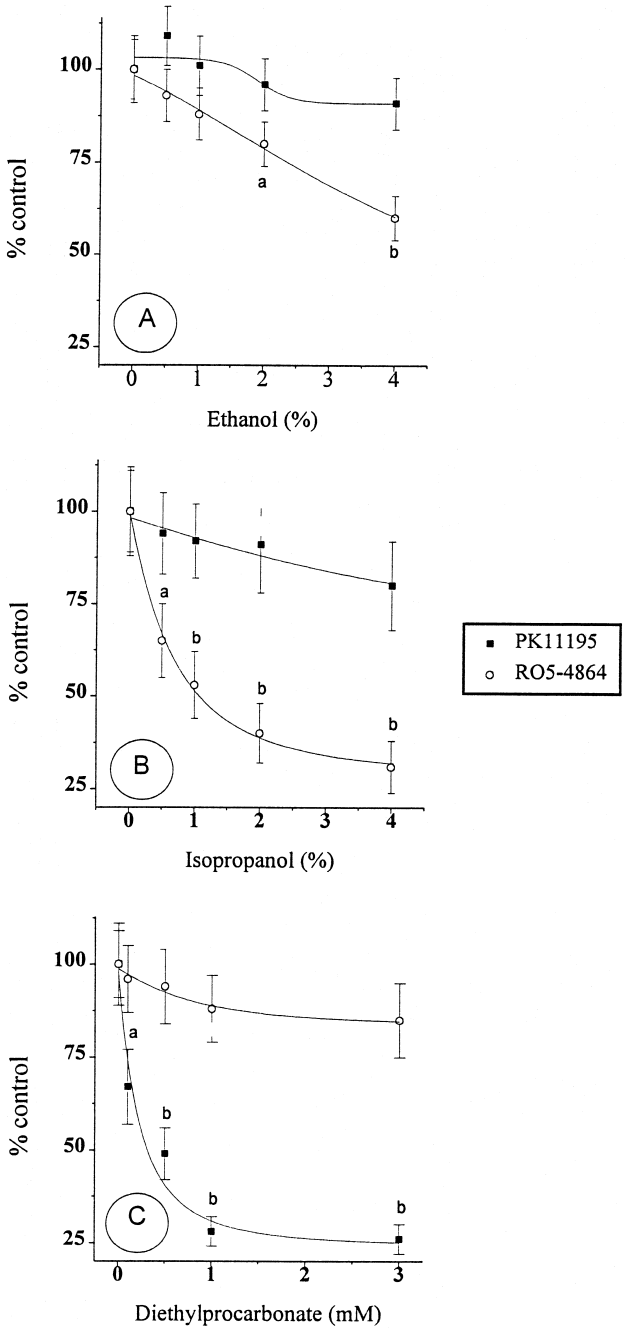

Fig. 6. Effects of increasing concentrations (0.5% to 4%) of isopropanol (A), ethanol (B) and histidine residue modification by diethylpyrocarbonate (0.1 mM to 3 mM) (C) on [^{3}H]PK11195 or [^{3}H]RO5-4864 specific binding. 65–75 μg protein equivalent of membranes were incubated for 120 min at 4°C in 50 mM Tris–HCl buffer (pH 7.4) containing 2 nM of either [^{3}H]PK11195 or [^{3}H]RO5-4864. Nonspecific binding was defined in the presence of either 2 μM PK11195 or 20 μM RO5-4864. For studying the effect of histidine residue modification, membrane preparations were incubated with the desired concentration of diethylpyrocarbonate for 5 min, before performing the binding assay. Values are given as mean $\pm$ SD ($n = 4$) of percent control values. Control values (100%) are 377 ± 47 ([^{3}H]PK11195 binding) and 40 ± 7 fmol/mg protein ([^{3}H]RO5-4864 binding). Statistics: $^aP < 0.05$ and $^bP < 0.01$ compared to control by ANOVA followed by Dunnett's multiple comparisons test.

3.7. Effect of histidine residue modification

Modification of histidine residues of the cortical membranes by incubating with diethyl-pyrocarbonate for 5 min decreased the binding of [^{3}H]PK11195 in a dose-dependent manner with a maximal inhibition of 70% at 1 mM (Fig. 6C). Increasing the incubation period (up to 20 min) and the concentration of diethylpyrocarbonate (up to 3 mM) had no further effect. In contrast, [^{3}H]RO5-4864 binding was resistant to histidine residue modification by diethylpyrocarbonate (Fig. 6C).

4. Discussion

Results of the present study demonstrate that both the benzodiazepine ([^{3}H]RO5-4864) and isoquinoline carboxamide ([^{3}H]PK11195) ligands for the peripheral-type benzodiazepine receptor bind to human brain homogenates with high affinity and manifest a similar pattern of regional distribution. However, they show differences in their kinetics, pharmacological profile and resistance to detergents. Maximal binding site densities for the isoquinoline ligand are ~ 3 fold higher than those of the benzodiazepine ligand in all brain regions studied. However, binding studied using either 2 nM [^{3}H] PK11195 or [^{3}H]RO5-4864 showed 6–8 fold higher density of PK11195 sites than RO5-4864 sites (Table 2). This discrepancy between the B_{max} values and single point data is due to the fact that the concentration of the ligands used (2 nm) for studying the regional distribution is nonsaturating. Peripheral-type benzodiazepine receptor is not a single protein receptor. Rather it is a multimeric complex in which the isoquinoline binding site is localized to a 18 kDa subunit and expression of the benzodiazepine site requires both the 18 kDa subunit as well as a 34 kDa voltage-dependent anion channel (VDAC) subunit (Garnier et al., 1994). In a study of mouse Leydig tumor cell mitochondrial preparations examined by transmission electron and atomic force microscopic procedures in order to evaluate the topography and organization of the peripheral-type benzodiazepine receptor, results indicated that the 18 kDa subunit was organized in clusters of 4–6 molecules (Papadopoulos et al., 1997). It was suggested that, in this way, the mitochondrial peripheral-type benzodiazepine receptor complex comprising the 18 kDa subunit and the VDAC functions as a pore whereby cholesterol is translocated to the inner mitochondrial membrane where it is metabolized to pregnenolone. The observation, in the present study of several-fold differences in maximal binding site densities between the isoquinoline and benzodiazepine ligands suggests that peripheral-type benzodiazepine receptor subunits in human brain are also organized as a heteromeric complex. These findings contrast observations in rat cerebral cortex (Awad and Gavish, 1987) and fish brain (Eshleman and Murray, 1989) in which comparable densities of binding sites for the iso-

quinoline and benzodiazepine ligand were observed. However, Itzhak et al. (1993, 1995) observed a ~1.5 to 2 fold higher density of binding sites for [^{3}H]PK11195 than [^{3}H]RO5-4864 in rat cortex and cultured rat astrocytes.

Other interesting differences between the human and rat brain preparations with reference to the peripheral-type benzodiazepine receptor include the observations that: (1) RO5-4864 displaces [^{3}H]PK11195 binding to the human peripheral-type benzodiazepine receptor with lower affinity than that observed in rat brain preparations, (2) RO5-4864 displaces [^{3}H]PK11195 binding in a monophasic manner in human brain preparations but in a biphasic manner in cultured astrocytes from rat brain (Itzhak et al., 1993) and (3) Both [^{3}H]PK11195 and [^{3}H]RO5-4864 binding are resistant to 1% ethanol in human brain. This concentration of ethanol significantly decreased the binding of both ligands to rat cortical astrocytes (Itzhak et al., 1993). The basis for these differences in affinities and other properties between human and rodent brain may lie in structural differences in peripheral-type benzodiazepine receptor proteins. In support of this possibility, cloning of the human 18 kDa subunit of the peripheral-type benzodiazepine receptor predicted an amino acid sequence that was missing the phosphorylation motif identified in the rodent sequence (Chang et al., 1992) suggesting that phosphorylation of the 18 kDa protein is not implicated in regulation of the human peripheral-type benzodiazepine receptor (Papadopoulos et al., 1997).

The human peripheral-type benzodiazepine receptor shows selective affinities for benzodiazepine and isoquinoline ligands. For example, the central benzodiazepine ligands, diazepam and clonazepam displace [^{3}H]RO5-4864 binding more potently than [^{3}H]PK11195 binding. These results were similar to those observed previously using rat cerebral cortex and cultured rat cortical astrocytes (Awad and Gavish, 1987; Itzhak et al., 1993). As expected, the novel peripheral-type benzodiazepine receptor isoquinoline site ligand $FGIN_{1-27}$ inhibited [^{3}H]PK11195 binding with high potency but had little effect on [^{3}H]RO5-4864 binding.

Peripheral-type benzodiazepine receptors are implicated in neurosteroid synthesis (Papadopoulos et al., 1997). The rate-limiting step in steroidogenesis is the delivery of cholesterol to cytochrome P-450 on the inner mitochondrial membrane and peripheral-type benzodiazepine receptors have been proposed to play a role in intramitochondrial cholesterol transport (Bernassau et al., 1993; Papadopoulos et al., 1997). The biosynthesis of neurosteroids begins with the enzymatic cleavage of the cholesterol side chain to form pregnenolone (Stocco and Clark, 1996). We therefore studied the effect of pregnenolone and its sulfate on [^{3}H]PK11195 and [^{3}H]RO5-4864 binding in human brain. Neither pregnenolone nor pregnenolone sulfate displaced either [^{3}H]PK11195 or [^{3}H]RO5-4864 binding suggesting that feedback inhibition of peripheral-type benzodiazepine receptor by neurosteroids is not an important regulatory mechanism, at least in human brain. None of several other steroids (progesterone, estradiol and testosterone) had any effect on the binding of [^{3}H]PK11195 or [^{3}H]RO5-4864 to human brain.

Differences between the isoquinoline and benzodiazepine sites on the human peripheral-type benzodiazepine receptor were also demonstrated by their differential susceptibility to various detergents as well as to histidine residue modification. In human brain, [^{3}H]PK11195 binding sites were significantly more resistant than [^{3}H]RO5-4864 binding sites to detergents such as Triton X-100, deoxycholic acid and Tween-20. These findings are similar to those previously reported in rat kidney membranes (Awad and Gavish, 1988). Similarly, in human brain, preincubation of the membranes with the thiol reagent dithiothreitol, resulted in a loss of both [^{3}H]PK11195 and [^{3}H]RO5-4864 binding sites. Similar effects of dithiothreitol were previously observed in rat brain membranes (Skowronski et al., 1987). Results of the present study clearly demonstrate that modification of the histidine residues by treating human brain membranes with diethylpyrocarbonate results in the loss of [^{3}H]PK11195 binding sites but not of [^{3}H]RO5-4864 binding sites. It was previously reported that diethylpyrocarbonate treatment inhibits [^{3}H]PK11195 binding to the membranes of rat kidney and mouse brain (Benavides et al., 1984; Skowronski et al., 1987). Other examples of differential modification of the isoquinoline and benzodiazepine binding sites of the rodent peripheral-type benzodiazepine receptor by various compounds has also been reported previously. Arachidonate, phospholipase A_2 and 4,4′-diisothiocyanotostilbene-2,2′-disulfonic acid led to modifications of [^{3}H]RO5-4864 binding without any significant effect on [^{3}H]PK11195 binding to rat kidney membranes (Lueddens and Skolnick, 1987; Havoundjian et al., 1986).

Exposure of primary rat cortical astroglial cultures to neurotransmitters including dopamine, serotonin, norepinephrine and GABA results in upregulation of [^{3}H]RO5-4864 binding sites without concomitant modification of [^{3}H]PK11195 binding sites (Itzhak and Norenberg, 1994). In contrast to these chronic effects of neurotransmitters, results of the present study demonstrate that none of the above neurotransmitters nor the excitatory amino acids glutamate and aspartate at concentrations of up to 5 μM had any significant effect on either [^{3}H]RO5-4864 or [^{3}H]PK11195 binding to human brain membranes. We also failed to observe any significant effect of other amino acids (glutamine, valine, leucine, isoleucine, tryptophan, phenylalanine, cysteine, methionine, histidine and arginine) on either [^{3}H]PK11195 or [^{3}H]RO5-4864 binding to human brain membranes.

Studies on the peripheral-type benzodiazepine receptor in human brain are sparse. Doble et al. (1987) analyzed the anatomical and subcellular distribution of [^{3}H]PK11195 binding sites in human brain. Their studies showed that the binding sites were distributed heterogenously but restricted

to the grey matter. Similar to the present study, Doble et al. (1987) also observed highest density of the PK11195 binding sites in forebrain structures. Awad and Gavish (1991) performed the only other study on human brain peripheral-type benzodiazepine receptors. The K_d for [^{3}H]PK11195 binding to human cortical membranes observed in the present study (2–3 nM) is similar to that reported by Awad and Gavish (1991). The authors reported that the binding sites for [^{3}H]RO5-4864 are 'barely detectable' in human cortex. The present study also revealed very low densities of [^{3}H]RO5-4864 binding sites in human brain which also confirms the results of Awad and Gavish (1991) showing the higher potency of PK11195 in displacing [^{3}H]RO5-4864 binding.

In summary, results of the present study demonstrate the presence of saturable, high affinity binding sites for [^{3}H]PK11195 and [^{3}H]RO5-4864 to the isoquinoline and benzodiazepine sites respectively on the human peripheral-type benzodiazepine receptor. A several-fold regional variation in densities of these sites was observed in human brain and the two sites were found to manifest distinct properties with respect to displacement of their ligands by benzodiazepines, to the effects of detergents and alcohols as well as to histidine residue modification. Unlike rodent brain, isoquinonine sites on the human peripheral-type benzodiazepine receptor outnumber the benzodiazepine site consistent with the existence of a hetoromeric complex. However, isoquinoline and benzodiazepine ligands are mutually competitive at nanomolar concentrations suggesting allosteric interaction between the two sites. Other differences between the rodent and human peripheral-type benzodiazepine receptor include the relative affinity of the isoquinoline site for RO5-4864 as well as the sensitivity of this site to alcohol. Such differences could result from distinct characteristics of the 18 kDa subunit of the peripheral-type benzodiazepine receptor between the two species.

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