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CHARACTERIZATION OF PERIPHERAL-TYPE BENZODIAZEPINE BINDING SITES FROM RAT AND PIG PANCREAS

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Abstract—The binding of [^{3}H]1-(2-chlorophenyl-*N*-methyl-1-methyl-propyl)-3-isoquinolinecarboxamide ([^{3}H]PK-11195) and [^{3}H]7-chloro-1,3-dihydro-l-methyl-5-(*p*-chlorophenyl)-2H-1,4-benzodiazepin-2-on ([^{3}H]Ro5-4864) to membrane preparations of pancreas was studied in the rat and pig. [^{3}H]PK-11195 bound with high affinity to rat and pig membrane preparations yielding maximal numbers of binding sites (B_{max}) of 2393 ± 160 and 777 ± 65 fmol/mg of protein, respectively, and equilibrium dissociation constant (K_d) values of 3.01 ± 0.25 and 3.9 ± 0.23 nM, respectively. [^{3}H]Ro5-4864 successfully labelled rat but not pig pancreatic membranes, yielding a K_d value of 6.45 ± 0.5 nM and a B_{max} value of 551 ± 43 fmol/mg of protein. Displacement studies showed a similar rank order of potency of various unlabelled ligands against both [^{3}H]Ro5-4864 and [^{3}H]PK-11195 binding to rat and pig membrane preparations (PK-11195 ≥ Ro5-4864 > diazepam > flunitrazepam ≫ flumazenil). These results suggest that [^{3}H]PK-11195 binds with high affinity and specificity to rat and pig pancreas and [^{3}H]Ro5-4864 binds with high affinity and specificity to rat but not pig pancreas.

Key words: peripheral benzodiazepine receptor; pancreas; [^{3}H]PK-11195; [^{3}H]Ro5-4864

BDZs† are one of the most widely used classes of drugs by virtue of their anxiolytic and anticonvulsant effects. It is established that a subset of these drugs binds to at least two classes of binding sites called central- and peripheral-type BDZ binding sites, respectively [1, 2]. CBR are mainly located within the central nervous system and are coupled to the $GABA_A$ receptor–chloride channel complex [3–5]. PBR differ from CBR in their lack of coupling to GABA receptors and in their ligand specificity. CBR exhibit high affinity to clonazepam but not to Ro5-4864 or PK-11195 (an isoquinoline carboxamide derivative). The reverse is true with regard to PBR, which exhibits high affinity to Ro5-4864 and PK-11195 but low affinity to clonazepam. Moreover, PBR binding sites are distributed both in the brain and in several peripheral tissues [6, 7]. In peripheral tissues the primary subcellular localization of PBR binding sites has been shown to be the mitochondrial compartment [8] and evidence suggests it is particularly localized in the outer mitochondrial membrane of rat adrenal cortex [8] and rat kidney [9].

Although PBR-induced activities have been described in a variety of biological systems [7] no clear and unequivocal functional role has yet been defined. Very recent studies employing the rat isolated pancreas and the rat isolated islets of Langerhans suggest that PBR may play a role in glucose-induced insulin secretion *in vitro*. In fact, Petit *et al.* [10] reported that glucose-induced insulin secretion *in vitro* was inhibited by Ro5-4864 and PK-11195, while clonazepam was without effect: the authors also suggest that Ro5-4864 may have a vasodilator effect on pancreatic vascular bleeding. With a view to clarify PBR's role on glucose-induced insulin secretion *in vitro*, as a preliminary step in this study the properties of membrane-bound PBR from rat pancreas, using [^{3}H]Ro5-4864 and [^{3}H]PK-11195 as specific ligands, were characterized. Moreover, the binding properties of [^{3}H]Ro5-4864 and [^{3}H]PK-11195 to membrane preparations of pig pancreas were studied to evaluate differences in PBR binding characteristics in different species.

MATERIALS AND METHODS

Materials. [^{3}H]PK-11195 (specific activity 86 Ci/mmol) and [^{3}H]Ro5-4864 (specific activity 85.4 Ci/mmol) were obtained from DuPont de Nemours (Germany). Diazepam, flunitrazepam and flumazenil were supplied by Hoffman-La Roche (Basel, Switzerland). All other compounds were purchased from chemical sources.

Membrane preparation. Male Sprague–Dawley rats (body weight ~200–250 g), were killed by decapitation. Adult male pigs were killed by electroshock in a local abattoir. Pancreas were removed immediately and frozen at −80° until used for binding studies. Pancreas were minced and suspended in 20 vol. of ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing soybean trypsin inhibitor (0.2 mg/mL), bacitracine (0.2 mg/mL), benzamidine (0.16 mg/mL), phenylmethylsulphonilfluoride (1 μg/

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† Abbreviations: BDZ, benzodiazepine; CBR, central benzodiazepine receptor; PBR, peripheral benzodiazepine receptor; GABA, γ-aminobutyric acid; PK-11195, 1-(2-chlorophenyl-*N*-methyl-1-methyl-propyl)-3-isoquinolinecarboxamide; Ro5-4864, 7-chloro-1,3-dihydro-1-methyl-5-(*p*-chlorophenyl)-2H-1,4-benzodiazepin-2-on.

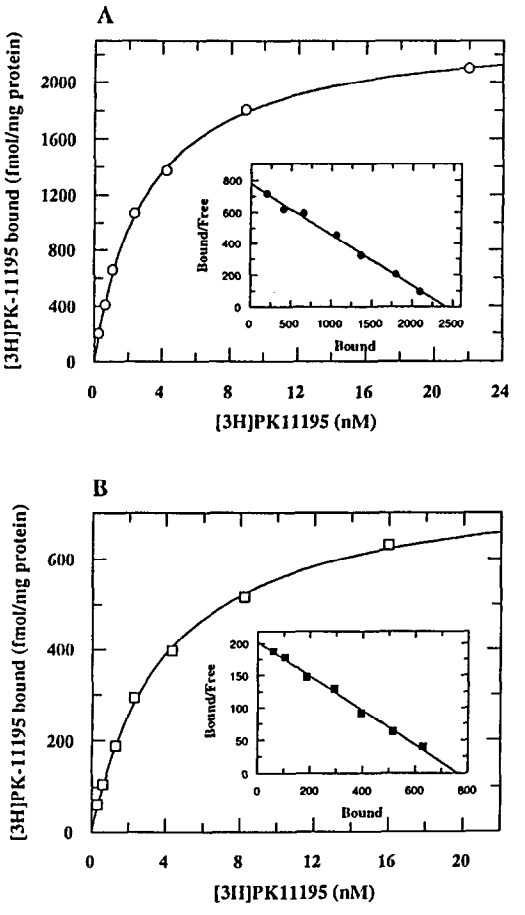

Fig. 1. Saturation curves [^{3}H]PK-11195 to rat (○) and pig (□) pancreatic membranes. Membranes were incubated for 90 min with seven different concentrations of radioligand ranging from 0.3 to 22 nM. Details of the binding procedure are described in Materials and Methods. Results are the means of three separate experiments with SEM less than 3%. Inset: Scatchard plots of saturation curves of [^{3}H]PK-11195 specific binding to rat (●) and pig (■) membranes.

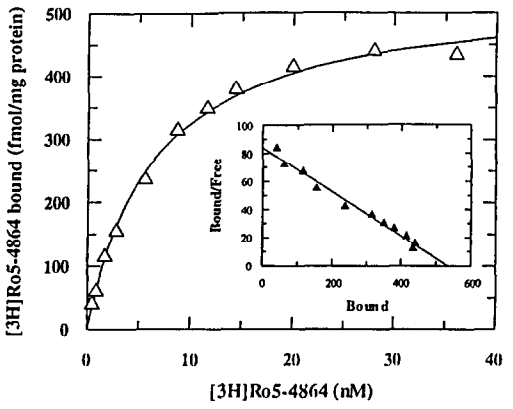

Fig. 2. Saturation curve of [^{3}H]Ro5-4864 to rat (△) pancreatic membranes. Membranes were incubated for 60 min with 11 different concentrations of radioligand ranging from 0.5 to 36 nM. Details of the binding procedure are described in Materials and Methods. Results are the means of three separate experiments with SEM less than 3%. Inset: Scatchard plot of saturation curves of [^{3}H]Ro5-4864 specific binding to rat (▲) membranes.

mL) and L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride (1 μg/mL), homogenized with an Ultra-turrax homogenizer, and centrifuged at 48,000 *g* for 15 min at 4°; the resulting pellets were suspended in 20 vol. of the same buffer, homogenized and centrifuged at 48,000 *g* for 15 min at 4°. The resulting crude membranes were suspended in 20 vol. of 50 mM Tris–HCl buffer, pH 7.4, without protease inhibitors and used in the binding assays.

Proteins were determined according to the method of Lowry *et al.* [11], using BSA as standard.

[^{3}H]PK-11195 *binding assay*. The binding assay to pancreatic membrane preparation was performed by incubating aliquots of membrane suspension (200 μg of protein) for 90 min at 0° in 500 μL of 50 mM Tris–HCl buffer, pH 7.4, containing 0.35 nM [^{3}H]PK-11195. Non-specific binding was defined as the binding of [^{3}H]PK-11195 in the presence of 1 μM unlabelled PK-11195 [12]. Saturation analysis of [^{3}H]PK-11195 binding sites was performed on the membrane receptor preparation using 0.3–22 nM [^{3}H]PK-11195.

[^{3}H]Ro5-4864 *binding assay*. The binding assay was carried out by incubating aliquots of the membrane fraction from rat pancreas (400 μg of protein) at 0° for 60 min in 500 μL of Tris–HCl buffer, pH 7.4, containing 2.5 nM [^{3}H]Ro5-4864; non-specific binding was conducted using 10 μM unlabelled Ro5-4864 [13]. Saturation analysis of [^{3}H]Ro5-4864 binding sites was performed on the membrane receptor preparation using 0.5–36 nM [^{3}H]Ro5-4864.

For both radioligands investigated, samples were filtered under vacuum over GF/C filters after the incubation time and washed three times with 5 mL of ice-cold Tris buffer. Filters were placed in vials containing 5 mL of Ready Protein Beckman scintillation cocktail and were counted for radioactivity using an LS 1800 scintillation counter.

For displacement studies, drugs were prepared as fresh solutions from ethanol stock solutions and were included in the reactions as indicated. The final concentration of ethanol was less than 1% in the incubation medium and did not interfere with binding.

Statistical analysis. Statistical analysis and curve fitting were carried out on an IBM compatible personal computer using the Grafit program.

RESULTS

Binding of [^{3}H]PK-11195 *and* [^{3}H]Ro5-4864 *to rat and pig pancreatic membranes*

Membrane preparations of rat and pig pancreas were incubated in the presence of increasing concentrations of [^{3}H]PK-11195 (0.3–22 nM). The specific binding (i.e. the difference between the binding in the absence of unlabelled PK-11195 and the binding in the presence of PK-11195), was found

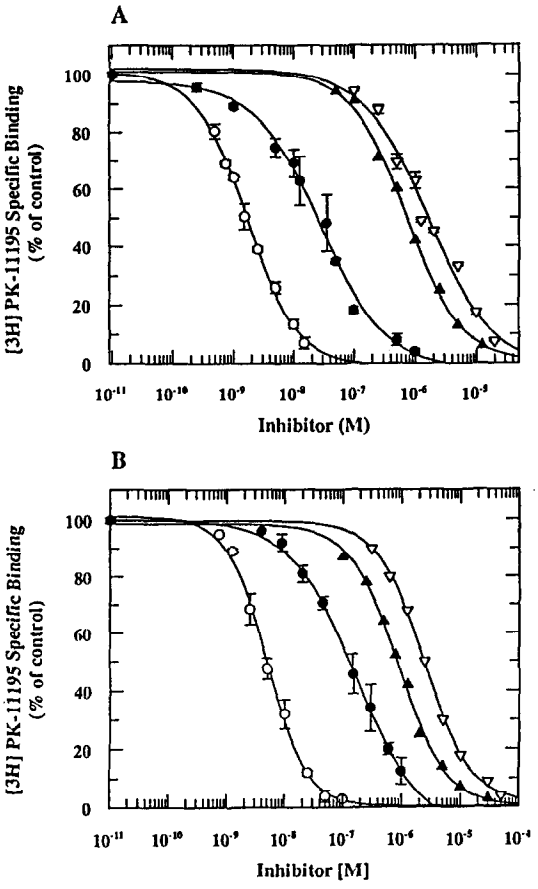

Fig. 3. Competition for specific [^{3}H]PK-11195 binding to the PBR. Representative data from competition analysis are shown for rat (A) and pig (B) membrane receptors. Eight to 10 different concentrations of PK-11195 (○), Ro5-4864 (●), diazepam (▲) and flunitrazepam (▼) were used to inhibit [^{3}H]PK-11195 binding to PBR. Binding assays were conducted with 0.35 nM [^{3}H]PK-11195. The data were plotted as the percentage of the binding seen in membranes in the absence of inhibitor (control) and represent the mean ± SEM of four independent determinations. Details of the binding assays are described in Materials and Methods. Average estimated K_i values were calculated from IC_{50} values using the Cheng and Prusoff equation (1973). K_i (in nm) obtained for PK-11195, Ro5-4864, flunitrazepam and diazepam were 1.48 ± 0.10, 23.30 ± 1.80, 1400 ± 115 and 636.20 ± 38, respectively, for rat membranes and 4.42 ± 0.34, 110 ± 9, 2330 ± 190 and 851 ± 48, respectively, for pig membranes.

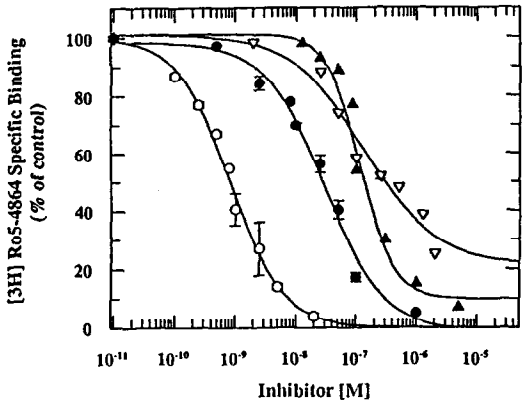

Fig. 4. Competition for specific [^{3}H]Ro5-4864 binding to the PBR. Representative data from competition analysis are shown for the rat membrane receptor. Eight different concentrations of PK-11195 (○), Ro5-4864 (●), diazepam (▲) and flunitrazepam (▽) were used to inhibit [^{3}H]Ro5-4864 binding to the PBR. Binding assays were conducted with 2.5 nM of [^{3}H]Ro5-4864. The data were plotted as the percentage of binding seen in membranes in the absence of inhibitor (control) and represent the mean ± SEM of four independent determinations. Details of the binding assays are described in Materials and Methods. Average estimated K_i values were calculated from IC_{50} values using the Cheng and Prusoff equation (1973). K_i (in nm) obtained for PK-11195, Ro5-4864, flunitrazepam and diazepam were 0.595 ± 0.03, 17.87 ± 1.30, 270 ± 13 and 103 ± 8.20, respectively.

to be saturable, whereas non-specific binding increased linearly with the concentration of tritiated ligand and was less than 15% of total binding at 0.35 nM [^{3}H]PK-11195. [^{3}H]PK-11195 bound with high affinity to both rat and pig membranes. Scatchard analysis of saturation curves yielded a single straight line for both species (Fig. 1A and B) suggesting the presence of a homogeneous population of binding sites. K_d values for [^{3}H]PK-11195 in rat and pig membranes were 3.01 ± 0.25 and 3.90 ± 0.23 nM, respectively, and B_{max} values were 2393 ± 160 and 777 ± 65 fmol/mg protein for rat and pig, respectively.

No specific binding of [^{3}H]Ro5-4864 was detected in pig pancreatic membranes. In contrast, [^{3}H]Ro5-4864 (0.5–36 nM) successfully labelled rat pancreatic membranes. The saturation curve demonstrated that the specific binding was saturable, whereas non-specific binding increased linearly with the concentration of tritiated ligand and was less than 30% of total binding at 2.5 nM [^{3}H]Ro5-4864. Scatchard analysis of the saturation curve exhibited a K_d value of 6.45 ± 0.50 nM and a B_{max} value of 551 ± 43 fmol/mg protein (Fig. 2).

Displacement studies

Pharmacological characterization of the PBR of rat and pig pancreatic membranes was determined using 1,4-BDZs and PK-11195 to compete for specific [^{3}H]PK-11195 binding.

In rat and pig pancreatic membrane preparations PK-11195 and Ro5-4864 showed nanomolar potencies as competitors for binding sites over the range of concentrations studied. Diazepam and flunitrazepam were less effective in both preparations: flumazenil, which binds with high affinity to the CBR, was not able to displace [^{3}H]PK-11195 from their binding sites over the range of concentrations studied. Displacement curves were obtained in the presence of 0.35 nM [^{3}H]PK-11195: the estimated dissociation constants (K_i) for each competing ligand were calculated from these data and are presented in Fig. 3A and B.

The potency of PK-11195 and 1,4-BDZs in displacing [^{3}H]Ro5-4864 from rat pancreatic membranes was also tested. The rank order of potency was PK-11195 > Ro5-4864 > diazepam > flunitrazepam ≫ flumazenil for rat pancreatic membranes. The estimated K_i values for each competing ligand were calculated from these data and are presented in Fig. 4.

DISCUSSION

The present study demonstrates the presence, in rat pancreas, of binding sites for [^{3}H]Ro5-4864 and [^{3}H]PK-11195, both selective ligands for PBR. The binding of both [^{3}H]Ro5-4864 and [^{3}H]PK-11195 was saturable and maintained an affinity value to PBR similar to that shown in the central nervous system and other peripheral organs. The binding was reduced by drugs having an affinity for PBR (such as unlabelled Ro5-4864 and PK-11195) but was not modified by flumazenil, a selective CBR ligand. Classical BDZs, such as diazepam and flunitrazepam, were less effective in PBR sites present in the pancreas compared to other peripheral tissues.

Rat pancreatic tissue had been included in a previous study on the presence of PBR in endocrine organs, low levels of [^{3}H]Ro5-4864 binding having been demonstrated [14].

The density of binding sites found in the rat pancreas labelled by [^{3}H]Ro5-4864 in the present study was substantially similar to that reported for rat pancreas by De Souza *et al.* [14], (54.6 ± 6.2 compared to 68.5 ± 3.4 fol/mg of protein at 0.64 nM of [^{3}H]Ro5-4864). On the contrary, to the best of the authors' knowledge, this is the first study showing the presence of binding sites for [^{3}H]PK-11195 in the pancreas.

Previous studies have shown species- and tissue-specific differences in the kinetic and pharmacological parameters of PBR, suggesting, in some cases, the existence of multiple receptor subtypes [15, 16]. These results are in agreement with species difference observed by those authors for other tissues. In fact, the PBR identified in the pig pancreas are characterized by an affinity for [^{3}H]PK-11195 in the nanomolar range. Displacement studies of [^{3}H]PK-11195 by unlabelled Ro5-4864 suggest that a large proportion of the binding marked by [^{3}H]PK-11195 has a low affinity for Ro5-4864 (K_i = 110 nM). Chemical BDZs displaced [^{3}H]PK-11195 binding with low affinity and no interaction by flumazenil was shown. In contrast to the results obtained in rat, it was not possible to detect the presence of a binding site for [^{3}H]Ro5-4864 in pig pancreas. The results obtained here do not provide any indication as to the possible physiological role of PBR in the pancreas. Further biochemical and functional studies are needed to clarify the correlation between the physiological effects reported by Petit *et al.* [10] and the presence of [^{3}H]PK-11195 and [^{3}H]Ro5-4864 specific binding sites in rat pancreas reported in these results.

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