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Characterization of Peripheral Benzodiazepine Receptors in Purified Large Mammal Pancreatic Islets

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ABSTRACT. In this work, we evaluated the biochemical properties of peripheral benzodiazepine receptors (PBRs) in the porcine endocrine pancreas and their role in insulin release. Binding of [^{3}H]1-(2-chlorophenyl-N-methyl-1-methyl-propyl)-3-isoquinolinecarboxamide ([^{3}H]PK-11195), a specific ligand of PBRs, to islet membranes was saturable and Scatchard's analysis of saturation curve demonstrated the presence of a single population of binding sites, with a dissociation constant (K_d) value of 4.75 ± 0.70 nM and a maximum amount of specifically bound ligand (B_{max}) of 4505 ± 502 fmol/mg of proteins. The pharmacological profile of PBRs was determined as the ability of PK-11195 and several benzodiazepine compounds to displace [^{3}H]PK-11195 from these binding sites. The rank order of potency yielded the following affinity results: PK-11195 > 7-chloro-1,3-dihydro-1-methyl-5-(p-chlorophenyl)-2H-1,4-benzodiazepine-2-on (Ro 5-4864) > diazepam ≥ flunitrazepam ≫ flumazenil. Secretion studies demonstrated that PK-11195 (1 and 10 μM) and Ro 5-4864 (10 and 50 μM) significantly potentiated insulin secretion from freshly isolated porcine islets at 3.3 mM glucose. This potentiating effect was not observed at 16.7 mM glucose concentration nor by the addition of clonazepam. These results show the presence of PBRs in purified porcine pancreatic islets and suggest an implication of PBRs in the mechanisms of insulin release. BIOCHEM PHARMACOL 51;11:1437–1442, 1996.

KEY WORDS. peripheral benzodiazepine receptor; porcine islets; insulin release; [^{3}H]PK-11195

BDZs§ were shown to exert their classical clinical action by interaction with receptors present in the GABA/chloride ion channel macromolecular complex. A second type of recognition site was discovered soon after, in the peripheral tissue and in the brain [1–4]. This second class of benzodiazepine peripheral binding site is not linked to the GABA receptor/chloride ion channel complex [1, 3], and differs from the CBRs in physiological function and pharmacological sensitivity [1, 5–7].

Several synthetic compounds, including quinolines, imidazopyridines, and benzodiazepines, reversibly interact with PBRs. BDZs have only low affinity (micromolar dissociation constants) in many species, with the notable exception of most rodents. 1,4 BDZs, such as flunitrazepam, diazepam, Ro 5-4864, and clonazepam, were initially used to characterize rat PBRs, which show a unique rank order of benzodiazepine affinity (Ro 5-4864 > diazepam ≥ flunitrazepam ≫ clonazepam). Conversely, rat CBRs [1–3, 8, 9] show the opposite rank order of benzodiazepine potency, with clonazepam having the highest affinity.

PBRs of most non-rodent species have only moderate to low affinity for BDZs. Despite the species variations in PBRs observed for 1,4-BDZs affinity, PK-11195, an isoquinoline carboxamide, has high affinity for PBRs in all species and is specific for these receptors [7, 10–12]. Molecular masses obtained after radiation analysis using irreversible ligands, such as [^{3}H]-flunitrazepam, [^{3}H]-AHN086, and [^{3}H]PK-14105, demonstrated that the former compounds radiolabel two proteins with molecular weight ranging from 30–35 KDa [13–15]. These proteins were identified as VDAC and ANC [16]. [^{3}H]PK-14105 specifically radiolabels the 17–19 KDa protein (IBP) [17, 18] that is an integral membrane protein coincident with the receptor in all species [5, 19, 20]. These proteins (VDAC, ANC, IBP) may form a heteromeric receptor complex.

To date, the physiological role of PBRs has not been finally determined, although some physiologic effects for PBR ligands have already been characterized, such as inhibition of cellular proliferation [21], induction of cellular differentiation [22, 23], and alteration in the release of several hormones [24]. Because the highest receptor densities occur in mitochondria, these organelles have been the target of several investigations. The ability of various compounds to inhibit mitochondrial respiration is well correlated with their affinity for PBRs [25]. These receptors are

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§ *Abbreviations:* BDZs, benzodiazepines; CBRs, central benzodiazepine receptors; PBRs, peripheral benzodiazepine receptors; GABA, γ-aminobutyric acid; PK-11195, 1-(2-chlorophenyl-N-methyl-1-methyl-propyl)-3-isoquinolinecarboxamide; Ro 5-4864, 7-chloro-1,3-dihydro-1-methyl-5-(p-chlorophenyl)-2H-1,4-benzodiazepin-2-on; VDAC, voltage-dependent anion channel; ANC, adenine nucleotide carrier; HBSS, Hanks' balanced salt solution; FCS, fetal calf serum.

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particularly concentrated in the mitochondria of stereoidogenic tissues and cells, where they may facilitate intramembrane cholesterol transport from the outer to the inner mitochondrial membrane, the rate-limiting step in steroid biosynthesis [26–29].

In a previous study, we demonstrated the presence of PBRs in pig pancreas and investigated their biochemical and pharmacological properties using selective radioligands [30]. Because PBRs are frequently associated with hormonal secretion control, in this study, we prepared pure islets of Langherans from the pig pancreas to investigate the properties of [^{3}H]PK-11195 binding sites and the implication of PBRs in the mechanisms of insulin release.

MATERIALS AND METHODS

Materials

[^{3}H]PK-11195 (specific activity 86 Ci/mmol) was obtained from DuPont de Nemours (Germany). HBSS, FCS, collagenase type XI, and Histopaque were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Eurocollins solution was from Fresenius, Runcorn, U.K. and insulin radioimmunoassay was from Sorin (Saluggia, Italy). Diazepam, flunitrazepam, clonazepam, and flumazenil were supplied by Hoffman-La Roche (Basel, Switzerland). All other compounds were purchased from chemical sources.

Preparation of the Islets

The islets were prepared by collagenase digestion and density gradient purification from the pancreases obtained from market-age pigs. The isolation procedure was similar to the method previously described [31]. The splenic lobe was cannulated and the digestion solution (collagenase type XI, 3900 units/mL, dissolved in 300 mL HBSS, with 2% FCS) was slowly injected to distend the tissue. After distension, the gland was placed into a 500-mL glass beaker, and the digestion solution not used for distension was added to the beaker. This was loaded into a shaking water bath at 37°C, activated at 120 revolutions per min. After 15 to 20 min, the pancreas was shaken with forceps for 30 sec; then, the digestate was filtered through 300 and 90 μm mesh stainless steel filters in sequence. The solution passed through the filters and the tissue entrapped on the 300-μm filter mesh were again placed into the water bath for further digestion. The tissue remaining on the 90-μm mesh filter was washed with 250 mL Eurocollins solution at 4°C and was left to settle for 20 min. The same procedure of filtration, washing, and settling in the Eurocollins solution was repeated at approximately 30 and 40 min from the beginning of the incubation. For the purification, the digestate was pelleted at 400 × *g* for 2 min at 4°C. Then, 1 mL of tissue was loaded into 50 mL plastic conicals and resuspended in 13 mL of Histopaque 1.077. This layer was topped with 10 mL HBSS, 2% FCS. After centrifugation at 800 × *g* for 5 min at 4°C, the islets were recovered at the interface between the Histopaque and the HBSS layer. Finally, the islets were washed with HBSS, 2% FCS, by centrifugation at 800 × *g* for 2 min at 4°C and resuspended in CMRL 1066 tissue culture medium. Islet yield and purity were estimated as previously described [31, 32].

Preparation of Islet Membranes

Membrane preparations were accomplished as previously described [30]. In brief, the islets were suspended in 20 vol. of ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing soybean trypsin inhibitor (0.2 mg/mL), benzamidine (0.16 mg/mL), phenilmethilsulfonilfluoride (1 μg/mL), and L-lChloro-3-(4-tosiylamido)-7-amino-2-heptanone hydrochloride (μg/mL) (T buffer), homogenized in an ultraturrax homogenizer, and centrifuged at 48,000 × *g* for 15 min at 4°C.

The resulting pellet was washed in 20 volumes of T buffer, homogenized, and centrifuged at 48,000 × *g* for 15 min at 4°C. Finally, the pellet was suspended in 10 vol. of 50 mM Tris-HCl buffer, pH 7.4 without protease inhibitors (T_1 buffer) and used in the binding assay.

Binding Studies

The binding assay of [^{3}H]PK-11195 was carried out as detailed in reference [30], by incubating aliquots of the membrane suspension (0.05 mg of proteins) at 0°C for 90 min in 500 μL of T_1 buffer containing 0.4 nM [^{3}H]PK-11195, either in the presence or absence of unlabeled PK-11195 (1 μM). The Scatchard analysis was performed using 0.5 to 38 nM labelled PK-11195. The assay was terminated by filtration through Whatman GF/C glass fiber filters under suction. After washing 3 times with 5 mL of ice-cold T_1 buffer, radioactivity was counted in 4 mL of Ready-Safe Beckman scintillation cocktail on a Packard 1600 TR scintillation counter.

The displacement studies were performed using fresh solutions of the following benzodiazepines: diazepam, flunitrazepam, flumazenil, and Ro 5-4864. Curve fitting was accomplished on an IBM compatible personal computer using the Grafit program [33].

Secretion Studies

The effects of both PK-11195 and Ro 5-4864 on insulin release from purified porcine islets was evaluated within 24 hr from islet isolation. The details of the insulin secretion study procedures used in our laboratory have been published previously [31]. For the purpose of this study, batches of 10 to 15 hand-picked islet equivalents (150 μm sized islets) were preincubated for 45 min at 37°C in Krebs Ringer bicarbonate solution, pH 7.4, containing 0.5% albumin and 3.3 mM glucose. Successively, the medium was removed and replaced with the Krebs Ringer bicarbonate solution, containing either 3.3 or 16.7 mM glucose, with or without the addition of 1 or 10 μM PK-11195, or 10 or 50 μM Ro 5-4864. To evaluate whether or not the central

acting benzodiazepine clonazepam could have some effect on insulin release, 10 and 50 μM clonazepam was added in the incubation medium in some experiments, at both glucose concentrations. At the end of 60 min incubation at 37°C, aliquots of the medium were removed for insulin radioimmunoassay.

RESULTS

At the end of the isolation procedure, the porcine islets were morphologically intact with purity of 70% or higher.

Membrane preparations from the purified islets were incubated in the presence of increasing concentrations of [^{3}H]PK-11195 as described in Materials and Methods and the Scatchard analysis obtained from the saturation curve yielded a single straight line, suggesting the presence of a homogeneous population of binding sites (Fig. 1). The K_d value of the compound was 4.75 ± 0.70 nM and the B_{max} value was 4505 ± 502 fmol/mg of proteins.

The pharmacological characterization of [^{3}H]PK-11195 binding sites on porcine islet preparations was determined using 1,4-BDZs and PK-11195. All the competing drugs have monophasic inhibition curves, with Hill coefficients close to unity (Table 1). The tritiated ligand was effectively displaced by low concentrations of antagonist PK-11195, and the agonist Ro 5-4864 was effective at 101 nM concentration. [^{3}H]PK-11195 binding could be displaced by diazepam and flunitrazepam, although with low effectiveness, whereas flumazenil, a selective ligand of central benzodiazepine receptors, was unable to displace [^{3}H]PK-11195 from the binding sites.

TABLE 1. Specificity of [^{3}H]PK-11195 binding in pig islet membrane preparations

Compound	K_i (nM)
PK-11195	2.4 ± 0.20
Ro 5-4864	101 ± 13
Diazepam	910 ± 63
Flunitrazepam	3400 ± 280
Flumazenil	>20,000

For each displacement curve, we used [^{3}H]PK-11195 (final concentration 0.4 nM) and 5 to 8 concentrations of displacers were examined. Each point is the mean ± SE of 4 determinations. Average estimated K_i (inhibition constant) values were calculated from IC_{50} (concentration inhibiting 50%) values using the Cheng and Prusoff equation [34].

The results of insulin release at 3.3 mM glucose level are detailed in Table 2. Compared to the secretion with glucose

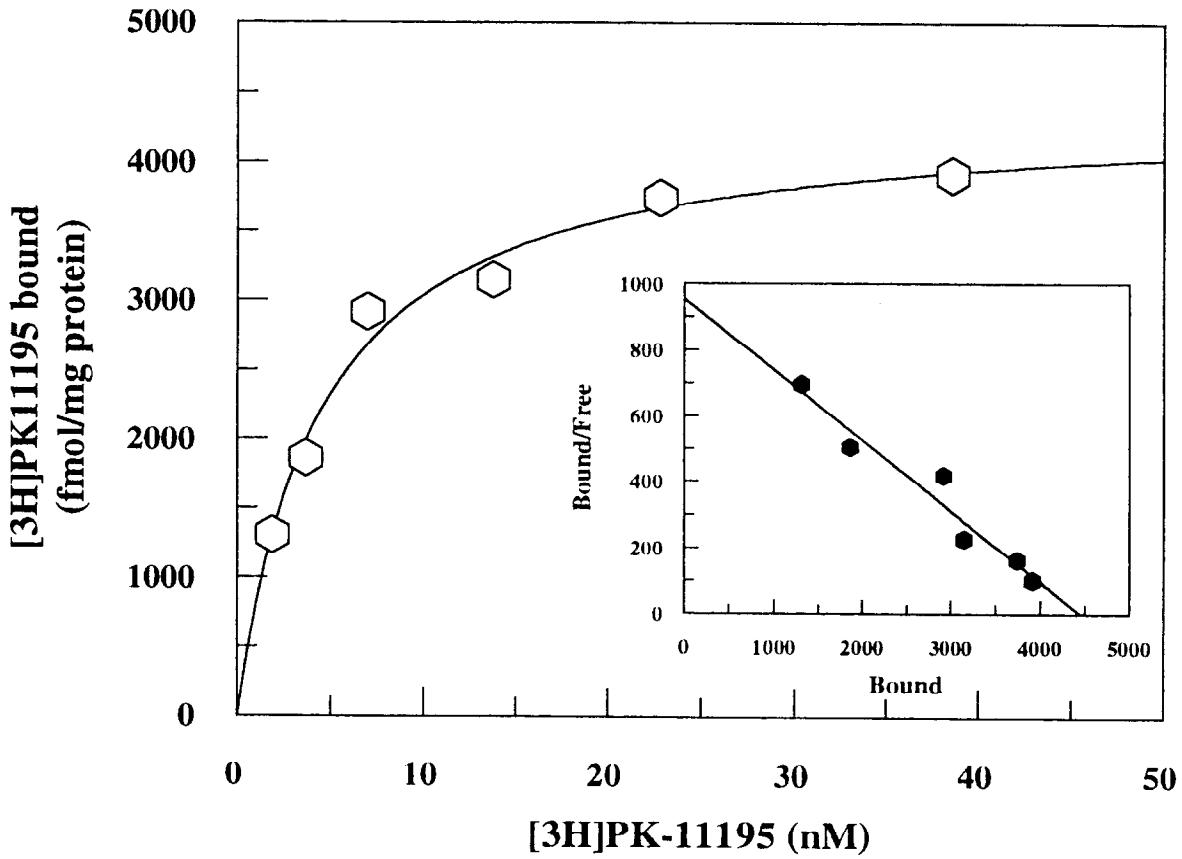

FIG. 1. Saturation curve of [^{3}H]PK-11195 to pig (○) islet membranes. Membranes were incubated for 90 min with 7 different concentrations of radioligand ranging from 0.5 to 38 nM. Details of the binding procedure are described in Materials and Methods. Results are the means of 3 separate experiments with SE less than 3%. Inset: Scatchard plot of saturation curve of [^{3}H]PK-11195 specific binding to pig (●) membranes.

TABLE 2. Insulin release (μU/islet equivalent/min) from isolated pig islets at 3.3 mM glucose, and the effect of PK-11195 and Ro 5-4864

	Secretagogue				
	3.3 G	3.3 G + PK 1	3.3 G + PK 10	3.3 G + Ro 10	3.3 G + Ro 50
Insulin release, mean	(6) 0.12	(5) 0.40*	(5) 0.21*	(5) 0.24*	(5) 0.44*
SE	0.03	0.02	0.02	0.04	0.08

Number of replicates in parentheses. 3.3 G, 3.3 mM glucose; PK 1, 1 μM PK-11195; PK 10, 10 μM PK-11195; Ro 10, 10 μM Ro 5-4864; Ro 50, 50 μM Ro 5-4864. *$P < 0.05$ vs 3.3 G by Bonferroni test.

alone, insulin output increased significantly with the addition of both PK-11195 and Ro 5-4864. The effect of the two compounds was similar, and no significant change was observed due to the different concentrations used in this study. When clonazepam was added to the medium, no significant change in insulin release was observed. The output of the hormone (μU/islet equivalent/min) at 3.3 mM glucose was 0.10 ± 0.02 without benzodiazepine and 0.09 ± 0.02 and 0.11 ± 0.03, respectively, with 10 and 50 μM clonazepam.

At 16.7 mM glucose concentration, insulin release was 0.40 ± 0.07 μU/islet equivalent/min (n: 6, $P < 0.05$ vs 3.3 mM glucose). The addition of 1 μM (n: 6) or 10 μM (n: 6) PK-11195 did not further increase hormone secretion (0.43 ± 0.04 and 0.43 ± 0.02 μU/islet equivalent/min, respectively). Similarly, when 10 or 50 μM Ro 5-4864 was added to 16.7 mM glucose, no significant change in insulin release (0.30 ± 0.09 and 0.33 ± 0.06 μU/islet equivalent/min, respectively) was observed compared to glucose alone. The addition of 10 and 50 μM clonazepam did not significantly affect insulin secretion, which was 0.31 ± 0.05 and 0.42 ± 0.07 μU/islet equivalent/min, respectively.

DISCUSSION

The actions of PBRs have been described in a variety of biological systems [35], some having direct effects, and others modulating the action of other substances. The PBR ligands seem to be involved in hormonal secretion control in various endocrine tissues, namely, the testis, the adrenal gland, and the pituitary gland [36]. However, no unifying effector mechanism has been discerned to account for the myriad of effects elicited by these receptors.

We previously reported the presence of [^{3}H]PK-11195 binding sites in porcine pancreas tissue [30]. In this study, we show a discrete localization of PBRs in the endocrine pancreatic tissue, and investigate the effect of two selective ligands for these receptors on insulin release from isolated porcine islets.

[^{3}H]PK-11195, a selective ligand for PBRs, bound to islet membranes in a saturable and reversible manner. From the data of saturation and competition studies with the radioligand, we deduce the presence of a single class of binding sites in porcine islets that shows the same profile of specificity as established PBR subtypes [37]. Similar findings have been previously described with other peripheral tissues of small and large mammals [38, 39].

It is interesting to note that the density of binding sites found in the islet membranes was substantially higher than that reported for whole tissue [30] and for acinar fraction (data not shown). This enrichment of PBR binding sites might be the expression of an implication of PBR in insulin secretion control. In fact, the results obtained in the present study demonstrate that PK-11195 and Ro 5-4864, classified by thermodynamic studies as agonist and antagonist for PBRs, respectively, significantly potentiated insulin release from isolated porcine islets in the presence of 3.3 mM glucose concentration. Petit and coworkers [40] showed an inhibitory effect of both compounds on rat islets. PK-11195 and Ro 5-4864 also have a similar effect in other physiological contexts, such as prolactin secretion [41] and cell growth [21, 42], suggesting that the functional significance of the thermodynamic classification is ambiguous. In addition, the differences as to the effects of PK-11195 and Ro 5-4864 on insulin release might be due to the varying species-specific characteristics of the PBRs [38, 39]. Under our experimental conditions, insulin release at 3.3 mM glucose was more markedly potentiated by 1 μM than 10 μM PK-11195. This is somewhat intriguing, because it is known that PK-11195 has only one binding site in all the species studied so far [38, 39]. Further studies are needed to explain this result.

To date, PBRs have been proposed as a ternary complex (constituated of 18 KDa protein, VDAC and ANC proteins) that forms a transport assembly that interacts with BDZ ligands mediating multiple cellular functions [43]. In several tissues, this complex has been shown to be associated with the outer mitochondrial membranes [44], site of oxidative metabolism. In this context, Snyder and coworkers [45] suggest a direct implication of PBRs in the control of the ATP transport rate. Because insulin secretion requires energy, we hypothesize a functional association between the possible role of PBRs in oxidative metabolism and the mechanisms of insulin secretion.

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