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Peripheral Benzodiazepine Receptors in Isolated Human Pancreatic Islets

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Abstract Peripheral benzodiazepine receptors have been shown in some endocrine tissues, namely the testis, the adrenal gland, and the pituitary gland. In this work we evaluated whether peripheral benzodiazepine receptors can be found in the purified human pancreatic islets and whether they may have a role in insulin release. Binding of the isoquinoline compound [^{3}H]1-(2-chlorophenyl-N-methyl-1-methyl-propyl)-3-isoquinolinecarboxamide ([^{3}H]PK-11195), a specific ligand of peripheral benzodiazepine receptors, to cellular membranes was saturable, and Scatchard's analysis of the saturation curve demonstrated the presence of a single population of binding sites, with an affinity constant value of 9.20 $\pm$ 0.80 nM and a maximum number of binding sites value of 8913 $\pm$ 750 fmol/mg of proteins. PK-11195 and 7-chloro-1,3-dihydro-1-methyl-5-(p-chlorophenyl)-2H-1,4-benzodiazepin-2-on (Ro 5-4864) significantly potentiated insulin secretion from freshly isolated human islets at 3.3 mM glucose. These results show the presence of peripheral benzodiazepine receptors in purified human pancreatic islets and suggest their role in the mechanisms of insulin release. J. Cell. Biochem. 64:273–277.

Key words: peripheral benzodiazepine receptor; [^{3}H]PK-11195; human islets; insulin release; Ro 5-4864

Two different classes of benzodiazepine recognition sites have been described. The central receptors (through which benzodiazepine drugs exert their classical clinical action) are part of the γ-aminobutyric acid (GABA)/chloride ion channel macromolecular complex present in the brain [Mohler et al., 1977]. A second type of benzodiazepine binding site has been discovered in the brain and in peripheral tissues, which is not linked to the GABA/chloride ion channel complex [Schoemaker et al., 1982a; Braestup and Squires, 1977] and differs from the central type receptors also in terms of pharmacological sensitivity [Braestup and Squires, 1977; Skowronski et al., 1987; Tallman et al., 1980].

In fact, the central benzodiazepine receptors (CBR) exhibit high affinity for clonazepam but not for 7-chloro-1,3-dihydro-1-methyl-5-(p-chlorophenyl)-2H-1,4-benzodiazepin-2-on (Ro 5-4864) or PK-11195 (an isoquinoline carboxamide derivative). The reverse is true with regard to peripheral benzodiazepine receptors (PBR), which exhibit high affinity for Ro 5-4864 and PK-11195 but low affinity for clonazepam.

PBR have been found also in some endocrine glands [De Souza et al., 1985; Benavides et al., 1983; Schoemaker et al., 1982b], but their physiological role is still unclear at this time. We have previously characterized the properties of PBR in the rat and pig pancreas [Giusti et al., 1994] and suggested a possible role of PBR in the mechanisms of insulin release from isolated porcine islets [Cosimi et al., 1994]. In the present study, we evaluated whether PBR can be found in purified, human pancreatic islets and whether they play a role in the mechanism of insulin release from the human endocrine pancreas.

METHODS

Materials

[^{3}H]1-(2-chlorophenyl-N-methyl-1-methyl-propyl)-3-isoquinolinecarb oxamide ([^{3}H]PK-11195) (specific activity 86 Ci/mmol) was ob-

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tained from DuPont de Nemours (Germany); Hanks' balanced salt solution (HBSS), fetal calf serum (FCS), Histopaque, and collagenase, type XI, were from Sigma Chemical Co. (St. Louis, MO); Eurocollins solution was from Fresenius (Runcorn, UK); insulin radioimmunoassay was from Sorin (Saluggia, Italy). Diazepam, flunitrazepam, 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 of five organ multidonors, obtained by our local organ procurement organization, AIRT. The age of the donors (two males, two females), ranged from 18–39 years. The isolation procedure was similar to the method previously described for the porcine and bovine pancreases [Marchetti et al., in press]. The splenic lobe was cannulated, and the digestion solution (collagenase, type XI, 3,900 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 into the beaker. This was loaded into a shaking water bath at 37°C, activated at 120 revolutions per minute. After 15–20 min, the pancreas was shaken with forceps for 30 s; 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 that remained on the 90 µm mesh filter was washed with 250 ml Eurocollins solution at 4°C, and it 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 pelletted 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. Islets yield and purity were estimated as previously described [Marchetti et al., 1994, in press].

Preparation of Islet Membranes

Membrane preparations were accomplished as previously described [Giusti et al., 1994]. In brief, the islets were suspended in 20 vol of ice-cold 50 mmol/l Tris-HCl buffer, pH 7.4, containing soybean trypsin inhibitor (0.2 mg/ml), benzamidine (0.16 mg/ml), phenilmethilsulfonil-fluoride (1 µg/ml) and L-1Chloro-3-(4-tosiyl-amido)-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 vol 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

Binding assay of [^{3}H]PK-11195 was carried out as previously described [Giusti et al., 1994] 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 0.5–32 nM labeled PK-11195. The assay was terminated by filtration through Whatman GF/C glass fiber filters under suction. After three washes with 5 ml of ice-cold T_1 buffer, the 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 [Leathebarrow, 1992].

Secretion Studies

The effects of both PK-11195 and Ro 5-4864 on insulin release from purified human islets was evaluated within 24 h from islet isolation. The details of the insulin secretion study procedures used in our laboratory have been pub-

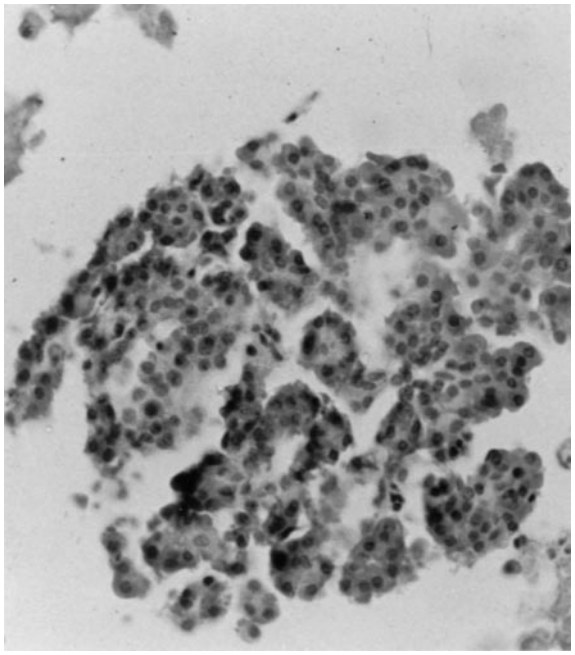

Fig. 1. Light photomicrograph of isolated human pancreatic islets.

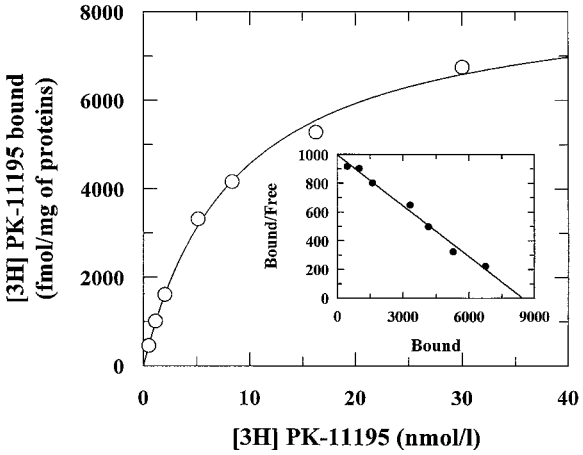

Fig. 2. Saturation curve of [^{3}H]PK-11195 to human (○) islet membranes. Membranes were incubated for 90 min with seven different concentrations of radioligand ranging from 0.5–32 nM. Details of the binding procedure are described in Methods. Results are the means of three separate experiments with SE less than 3%. **Inset:** Scatchard plot of the saturation curve of [^{3}H]PK-11195 specific binding to human (●) membranes.

lished previously [Marchetti et al., in press]. For the purpose of this study, batches of 10–15 handpicked islets 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 different concentrations of PK-11195 (25 nM to 10 µM) or Ro 5-4864 (25 nM to 50 µM). At the end of a 60 min incubation at 37°C, aliquots of the medium were removed for insulin radioimmunoassay.

RESULTS

At the end of the isolation procedure, the yield from the four glands processed for the purpose of this study was 1,987 ± 148 islet equivalents (150 µm sized islets) per gram of pancreatic tissue. The islet purity, as assessed by two different operators both on a phase microscope after dithizone staining of the tissue [Marchetti et al., 1994] and by light microscopy evaluation of semithin sections of the final preparations [Marchetti et al., in press] was estimated to be higher than 70% (Fig. 1).

When membrane preparations from the purified islets were incubated in the presence of increasing concentrations of [^{3}H]PK-11195, the Scatchard analysis of the saturation curve yielded a single straight line, suggesting the presence of a homogeneous population of binding sites (Fig. 2). The dissociation constant (Kd) of the compound was 9.20 ± 0.80 nM, and the maximum amount of specifically bound ligand (Bmax) was 8,913 ± 750 fmol/mg of proteins.

TABLE I. Specificity of [^{3}H]PK-11195 Binding in Human Islet Membrane Preparations*

Compound	Ki (nM)
PK-11195	3.56 ± 0.30
Ro 5-4864	170 ± 15
Diazepam	480 ± 43
Flunitrazepam	2,860 ± 250
Flumazenil	>20,000

*Five to eight concentrations of displacers were examined. Each point is the mean ± SE of four determinations. Average estimated Ki (inhibition constant) values were calculated from IC_{50} (concentration inhibiting 50%) values using the Cheng and Prusoff equation [1973].

Pharmacological characterization of PBR human islet preparations was determined using 1,4-benzodiazepines and PK-11195 to compete for specific [^{3}H]PK-11195 binding (Table I). The tritiated ligand was effectively displaced by low concentrations of antagonist PK-11195, while the agonist Ro 5-4864 was effective at a 170 nM concentration. [^{3}H]PK-11195 binding could be displaced by diazepam and flunitrazepam, but with low effectiveness, whereas flumazenil, a selective ligand of central benzodiazepine recep-

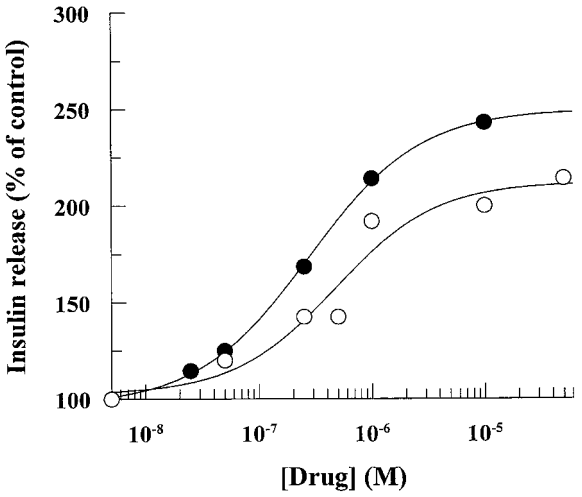

Fig. 3. Effects of PK 11195 and Ro 5-4864 on insulin release from purified human pancreatic islets incubated in the presence of 3.3 mM glucose. Five to seven different concentrations of PK 11195 (●) and Ro 5-4864 (○) were used to increase insulin secretion to human pancreatic islets. Details of the secretion studies are described in Methods. Data were plotted as drug concentration (on a logarithmic scale) vs. percentage of control. The values are the means of triplicate determinations with SEM less than 10%. This experiment was repeated twice with similar results.

tors, was unable to displace [^{3}H]PK-11195 from the binding sites.

At the 3.3 mM dextrose level the effects of PK-11195 and Ro 5-4864 on insulin release appeared concentration-dependent (Fig. 3). Compared to the secretion with glucose alone, insulin output increased significantly with the addition of both PK-11195 and Ro 5-4864. The EC_{50} values, calculated by the Grafit program, were 274 nM and 469 nM for PK-11195 and Ro 5-4864, respectively.

When clonazepam was added to the medium, no significant change on insulin release was observed. The output of the hormone (microunits/islet equivalent/minute) at 3.3 mM glucose was 0.07 ± 0.01 without benzodiazepine and 0.06 ± 0.02 and 0.09 ± 0.03 with 10 μM and 50 μM clonazepam, respectively.

At a 16.7 mM glucose concentration, insulin release was 0.21 ± 0.04 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 (respectively, 0.23 ± 0.04 and 0.20 ± 0.03 μU/islet equivalent/min). Similarly, when 10 or 50 μM Ro 5-4864 was added to 16.7 mM glucose, no significant change of insulin release (respectively, 0.21 ± 0.02 and 0.19 ± 0.04 μU/islet equivalent/min) was observed compared to glucose alone. The addition of 10–50 μM clonazepam did not significantly affect insulin secretion, which was 0.19 ± 0.04 and .23 ± 0.05 μU/islet equivalent/min, respectively.

DISCUSSION

Peripheral benzodiazepine receptors have been studied in human tissues mainly to evaluate their value as tumoural markers, and an association of PBR has been reported with tumoural processes of the prostate [Camins et al., 1994], glial tumors [Broaddus and Bennet, 1990], and human colonic adenocarcinoma [Katay et al., 1990]. There is also a growing body of data that supports an implication of PBR in immune function, since these high affinity binding sites were found on lymphocytes and granulocytes [Ruff et al., 1985; Lenfant et al., 1985].

Peripheral-type benzodiazepine recognition sites have been characterized also in various endocrine tissues, namely the testis, the adrenal gland, and the pituitary gland [De Souza et al., 1985; Benavides et al., 1983; Schoemaker et al., 1982b], where they seem to be involved, with a stimulatory effect, in hormonal secretion control.

In the present study for the first time we provide direct experimental evidence of the existence of PBR in human islets of Langherans. [^{3}H]PK-11195, a selective ligand for PBR, bound to islet membranes in a saturable manner. From the data of saturation and competition studies with the radioligand, we deduced the presence of a single class of binding sites that shows the same profile of specificity as the established PBR subtype [LeFur et al., 1983]. The high density of PBR in human islets (Bmax = 8,913 ± 750 fmol/mg of proteins) suggests that this receptor may be functionally associated with regulation of hormonal secretion. In this context, we investigated the effect of PK-11195 and Ro 5-4864 on insulin release from isolated islets. Thermodynamics studies of the binding of PK-11195 and Ro 5-4864 to the heart suggested antagonist properties of the former and agonist properties of the latter compound [LeFur et al., 1983]. In the present study, the two drugs were found to have similar effects on human islets by significantly potentiating insulin release at 3.3 mM glucose. PK-11195 and Ro 5-4864 have been shown to have similar insulinotropic actions also on porcine islets [Cosimi et al., 1994]. With rat islets both PK-11195 and Ro 5-4864 inhib-

ited insulin secretion [Petit et al., 1992], suggesting species differences in the pharmacological characteristics of PBR [Awad and Gavish, 1987]. Further studies are needed to fully elucidate the physiological role of these two compounds in in vivo models.

In numerous tissues, PBR have been shown to be associated with protein complexes of the outer mitochondrial membranes [Anholt et al., 1986; Snyder et al., 1990], sites of the oxidative metabolism. Recently, based on immunochemical studies, Snyder and coworkers [McEnery et al., 1992] identified the PBR with the mitochondrial protein of ATP-ADP transport (voltage-dependent anion channel/adenine nucleotide carrier), supporting the hypothesis of a direct implication of PBR in the control of the ATP transport rate. Since the insulin secretion process requires energy, we hypothesize a functional association between the possible role of PBR in oxidative metabolism and the mechanisms of insulin secretion.

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