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Two Cellular and Subcellular Locations for the Peripheral-type Benzodiazepine Receptor in Rat Liver

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ABSTRACT. Determination of ligand binding properties of the peripheral benzodiazepine receptor (PBBS) in liver, in hepatocytes, and in nonparenchymal cells demonstrated the presence of receptor-specific high-affinity binding in both hepatocyte and nonhepatocyte cells. Density gradient centrifugation showed that the high-affinity receptor in hepatocytes was localised to mitochondria, whereas in nonhepatocytes it was not mitochondrial, but with a possible biliary epithelial cell plasma-membrane location. Both receptors showed the peripheral-type specific high-affinity binding of PK 11195 and Ro5 4864 and could be photolabelled as 18 kDa proteins with [^{3}H]PK 14105. Immunocytochemistry showed the presence of acyl-CoA binding protein, a putative endogenous ligand for the receptor, in both cell locations. Some other properties of the PBBS were investigated in liver. Diphosphatidyl glycerol had a strong inhibitory effect on receptor binding in both liver and adrenal, with Ro5 4864 more sensitive to inhibition than PK 11195. However, whereas soybean lipid and phosphatidyl serine increased the binding of both ligands to adrenal receptor, these lipids had no effect on liver, suggesting that liver PBBS may differ from the well-characterised adrenal PBBS in some of its protein conformation. Modulators of mitochondrial respiration that also influence intermembrane contact site formation were found to elicit no marked stimulatory or inhibitory effects on PBBS ligand binding in liver, a result also found for adrenal mitochondria, suggesting that the extent of contact site formation does not influence ligand binding and that the hepatocyte receptor may not play a role in regulating mitochondrial respiration. These two cellular and subcellular locations of the PBBS in liver and the different effect of phospholipids compared to other peripheral tissues may be important for the role(s) of PBBS in liver and also for the multiple roles ascribed to the receptor and to peripheral-type benzodiazepine ligands. BIOCHEM PHARMACOL 51;10:1283–1292, 1996.

KEY WORDS. peripheral benzodiazepine receptor; DBI; ACBP; rat liver; mitochondria

The anxiolytic, sedative, and anticonvulsant effects of benzodiazepine are mediated through the central-type benzodiazepine receptor, which is associated with the neuronal γ-aminobutyrate (GABA)-gated chloride channel [1, 2]. A second type of benzodiazepine receptor is present in glial cells and in peripheral tissues [3], and is highly expressed in steroidogenic tissue [4]. Autoradiographic and immunocytochemical approaches have localised this PBBS† to discrete cell-types such as glia, epithelial cells of the choroid plexus and kidney, adrenal cortical cells, Leydig cells in the testis, and the bile duct epithelial cells of the liver [4, 5]. Subcellular fractionation studies have provided evidence for a mitochondrial outer membrane location in peripheral tissues [6–8], although a second location has been described in liver [7] and heart [9], and a cell surface fraction of PBBS binding has been reported in adrenals [10] and in testis [11].

The peripheral receptor is pharmacologically distinct from the central-type receptor and, in addition to binding benzodiazepines with different specificities, it also binds isoquinoline carboxamides such as PK 11195 with high affinity [12]. Although the binding of both classes of ligand to the PBBS is mutually exclusive at nanomolar concentrations, differential inhibitory effects of detergents [13], phospholipids, and thiol reagents [14–16] indicate that the binding sites for these ligands may not be identical. A third type of ligand for the peripheral receptor is DBI, an endogenous 10kDa polypeptide that has been purified from brain and from peripheral tissues and displaces the binding of ligands to both central- and peripheral-type benzodiazepine receptors [17, 18]. DBI is also known as ACBP in liver due to its ability to bind saturated acyl-CoA esters with chain lengths from 8 to 18 carbon atoms [19], and steroidogenesis activating factor (SAF) in adrenals due to its effect in increasing steroid production [20]. Immunocytological studies have revealed that DBI is expressed selectively in specialised cell types and that there is a correlation between the density and location of DBI and of binding sites in most

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† *Abbreviations:* PBBS, peripheral-type benzodiazepine receptor; Ro5 4864, 7-chloro1,3-dihydro-methyl-5-(*p*-chlorophenyl)-2H-1,4-benzodiazepine-2-one hydrochloride; PK 11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide; PK 14105, 1-(2-fluoro-5-nitrophenyl)-3-isoquinoline carboxylic acid; DBI, diazepam binding inhibitor; ACBP, acyl coenzyme A binding protein; ANC, adenine nucleotide carrier; VDAC, voltage dependent anion channel.

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tissues [18]. However, in liver, a high content of DBI was due to the diffuse presence of this protein in hepatocytes [18], whereas a study in our laboratory has located the receptor only in the bile duct epithelial cells [5].

An 18kDa isoquinoline carboxamide-binding protein has been isolated and cloned [21–23]; purification of the PBBS in a form that retained reversible ligand binding activity has suggested a close association of this 18kDa protein with the adenine nucleotide carrier of the mitochondrial inner membrane and the voltage-dependent anion channel (VDAC or porin) of the mitochondrial outer membrane [16]. *In vitro* reconstitution of a functional PBBS has provided evidence that the isoquinoline binding site is on an 18kDa subunit and expression of the benzodiazepine binding site requires both the 18kDa-protein and the voltage-dependent anion channel subunit [24]. It has been proposed that the receptor complex could occur at regions of intimate contact between the mitochondrial membranes [16]. A contact site location for the receptor has also been suggested, following observations that the stimulation of steroidogenesis by specific PBBS ligands and by DBI is due to an increase in the rate-limiting translocation of cholesterol from the outer to the inner mitochondrial membrane [25–27].

Because the liver is the only tissue in which distribution of the receptor and its endogenous ligand appear not to be coincident [5, 18], we characterised the binding of two specific ligands of the PBR in liver and in subpopulations of liver cells. High-affinity PBBS binding to an 18kDa protein was demonstrated in hepatocyte mitochondria and in a nonmitochondrial fraction of nonparenchymal cells. This is the first report that describes different, discrete subcellular locations for the peripheral benzodiazepine receptor in separate cell types in the same tissue. We, further, compared the properties of liver PBR to the receptor in other tissues, including a possible association of the peripheral benzodiazepine receptor with contact sites as a molecular complex. The effects of some potential modulators of binding were examined and several differences between the liver and other tissues were observed.

MATERIALS AND METHODS

Materials

[^{3}H]Ro5 4864 (86 Ci/mmol) and [^{3}H]PK 11195 (85.8 Ci/mmol) were purchased from N.E.N., Dreiech, Germany, [^{3}H]PK 14105 (87 Ci/mmol) was from C.E.A., Gif-sur-Yvette, France and 5-hydroxy[^{14}C]tryptamine (50 Ci/mmol) was from Amersham International. Ro5 4864 was purchased from Fluka (Gillingham, U.K.) and PK 11195 was a gift from Dr. A. Doble, Centre de Recherches de Gennevilliers, B.P. 158, 92231 Gennevilliers Cedex, France. The VECTASTAIN ABC kit was purchased from Vector Laboratories (Peterborough, U.K.). L-α-Phosphatidyl-L- serine (bovine brain), L-α-phosphatidylinositol (bovine brain), L-α-phosphatidylcholine,dimyristoyl, L-α-phosphatidylethanolamine,dimyristoyl, and L-α-phosphatidylcholine (L-α-lecithin) from soybean were purchased from Sigma (St. Louis, MO, U.S.A.). Emulsions of lipids were prepared by sonication (four 10-sec bursts with a Dawe soniprobe, Type 7532A, at a setting of 100W).

Determination of [^{3}H]ligand Binding to Mitochondrial Membranes

'Mitochondrial' preparations from rat adrenal, rat liver, and hepatocyte or nonparenchymal cells were prepared as described in [5], except that protein was determined by the method of Markwell *et al.* [28]. The final pellet was resuspended either in 50 mM Tris/HCl buffer, pH 7.4 and stored at −20°, or in the same buffer containing 1 mM EDTA and 0.32 M sucrose, and used immediately.

Adrenal, liver, hepatocyte, and nonparenchymal 'mitochondrial' fractions or 'mitochondrial' membranes (15–200 μg protein) were incubated with 1–2 nM [^{3}H]Ro5 4864 (30–90 Ci/mmol) or 1–2 nM [^{3}H]PK 11195 25–85.6 Ci/mmol) or, for saturation binding curves, 0.8–103 nM [^{3}H]Ro5 4864 (2.5 Ci/mmol) or 0.4–104 nM [^{3}H]PK 11195 (3.5 Ci/mmol). Specific/saturable binding was determined as described in [5] except that the incubation mixtures were filtered with a Brandel Cell Harvester. All assays were carried out in triplicate. Mean values for specific binding and free ligand concentration were fitted to equations describing a rectangular hyperbola or two rectangular hyperbolae by weighted nonlinear regression [29] using the computer program ENZFITTER (Elsevier-BIOSOFT, Cambridge, U.K.) [30].

Preparation of Liver Cells and Subcellular Fractionation

Rat liver hepatocytes were separated from nonparenchymal liver cells by the collagenase perfusion method described in [31] except that, instead of *in situ* perfusion of liver, single lobes of a freshly excised rat liver were cannulated and perfused. Crude 'mitochondrial' membrane fractions were prepared, as described above, from hepatocytes and from the white strands of biliary and connective tissue that remained after perfusion.

Isopycnic Density Gradient Centrifugation

The crude 'mitochondrial' fraction from hepatocytes (3.5 mL, 20 mg protein) or from nonparenchymal cells (3.5 mL, 18 mg protein) was layered on top of a continuous 1.0–1.6 M sucrose gradient (26 mL) over a 3-mL bed of 1.6 M sucrose and centrifuged at 97,000 *g* for 2 hr at 4°C. Samples (1 mL) were collected from the bottom of the gradient using a peristaltic pump and were stored at −20° until determination of binding and enzyme activities. Protein was estimated by the method of Markwell *et al.* [28]. Monoamine oxidase A (amine:oxygen oxidoreductase, E.C.1.4.3.4) was assayed as described [32] using 5-hydroxy[^{14}C]tryptamine (100 μM, 1 Ci/mmol) as substrate. Succinate dehydrogenase (succinate;(acceptor) oxidodreductase, E.C.1.3.99.1) was measured as in [33] and γ-glu-

tamyltransferase ((γ-glutamyl)-peptide:amino acid γ-glutamyl transferase, E.C. 2.3.2.2.) was measured using the IFCC Reference Method as described [34].

Photoaffinity Labelling with [^{3}H]PK 14105

Hepatocyte and nonparenchymal 'mitochondrial' membranes (2 mg protein), prepared as described above, were incubated with [^{3}H]PK 14105 (25 nM, 90 Ci/mmol) in a final volume of 1 mL in the absence (total labelling) or presence (nonspecific labelling) of PK 11195 (10 μM). Photoaffinity labelling and subsequent determination of the molecular size of the labelled proteins was carried out as described [5].

Preparation of Antiserum

ACBP was purified from rat liver as previously described [19]. This procedure involved acid- and heat-denaturation steps, delipidation, gel chromatography on Sephacryl S200 and Sephadex G50 columns, and reverse-phase HPLC. ACBP-containing fractions were identified by their ability to inhibit specific [^{3}H]PK 11195 binding to rat liver mitochondrial membranes. Freeze-dried, purified ACBP (50 μg protein) was dissolved in 750 μL PBS and emulsified with an equal volume of Freund's Complete Adjuvant. The emulsion was injected intradermally into 20 sites on the back of a male New Zealand White rabbit. Booster injections, prepared by mixing 750 μL antigen (as above) with 750 μL Freund's Incomplete Adjuvant, were injected intramuscularly 21 days and 31 days after the initial injection. Blood was collected by cardiac puncture 10 days after the second boost. The blood was left to clot at room temperature for 1 hr and then at 4°C overnight. Serum was decanted, centrifuged at 1000 *g* for 10 min and stored at −20° until further use.

Immunocytochemistry

Paraffin-embedded sections of rat liver were prepared as described [5], and immunocytochemical staining with anti-ACBP antiserum was carried out using the VECTASTAIN ABC kit as recommended by the manufacturers. Both polyclonal antiserum and preimmune serum were used at a dilution of 1/500.

PAGE and Western Immunoblotting

SDS/PAGE was performed by the method of Laemmli [35] on 15% or 20% (w/v) polyacrylamide gels. Protein was electrophoretically transferred from the gels to nitrocellulose sheets as described [5]. After treatment for 1 hr with a solution of 3% (w/v) BSA in 0.5 M NaCl/0.5% (v/v) Tween 20/20 mM Tris/HCl buffer, pH 7.5 (TTBS), the blots were incubated with either primary antiserum or preimmune serum (both diluted 1/100 in TTBS) at 4°C for 1 hr. Subsequent development of the blots was carried out using the VECTASTAIN ABC kit as recommended by the manufacturers, using chloronaphthol as the peroxidase substrate.

RESULTS AND DISCUSSION

Characterisation of PBR Ligand Binding in Liver

When rat liver tissue was enzymically dissociated into cells, an hepatocyte fraction was obtained, as well as a filamentous residue consisting of the white strands of biliary and connective tissue that remained after perfusion. High-affinity binding of [^{3}H]Ro5 4864 or [^{3}H]PK 11195 was found to 'mitochondrial' fractions from homogenates of the hepatocyte and nonparenchymal cells, with a similar binding capacity (B_{max} pmol/mg protein) in both fractions (Table 1). Lower-affinity binding was also found in some of the fractions. Better fits for the high-affinity binding sites were

TABLE 1. Equilibrium constants for binding of [^{3}H]Ro5 4864 and [^{3}H]PK 11195 to rat liver cells, hepatocytes, and nonparenchymal cells

	Ro5 4864		PK 11195	
	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)
High affinity:				
liver	0.7 ± 2	0.4 ± 0.3	0.7 ± 0.3	0.8 ± 0.1
hepatocytes	3.5 ± 1	1.5 ± 0.1	2.4 ± 1	1.2 ± 0.2
nonparenchymal	0.9 ± 0.5	1.2 ± 0.2	1.3 ± 0.3	3.1 ± 0.4
Low affinity:				
liver	0.2 ± 0.3	4.5 ± 4	0.3 ± 0.2	38 ± 5
hepatocytes	–	–	1 ± 2	15 ± 25
nonparenchymal	15,000 ± 300,000	41,000 ± 806,000	0.4 ± 0.4	13 ± 9

Crude mitochondrial membranes from rat liver (200 μg protein), hepatocytes, and nonparenchymal cells (100 μg protein) were assayed for saturable binding of [^{3}H]Ro5 4864 (0.8–103 nM, 2.5 Ci/mmol) and [^{3}H]PK 11195 (0.4–104 nM, 3.5 Ci/mmol). The equilibrium binding constants (K_d) and the maximal binding capacities (B_{max}) were calculated by fitting the data to the equations describing one or two rectangular hyperbolae by weighted nonlinear regression [29, 30]. The results are the average of 2 experiments (± range) and data from the best fits are shown.

found when the data was fitted to the equation describing two rectangular hyperbolae (i.e. both high- and low-affinity binding sites). However, the low-affinity binding was not further characterised due to the very high concentrations of radioligand required. The apparently higher levels of high-affinity [^{3}H]PK 11195 over [^{3}H]Ro5 4864 binding (approximately 2-fold) in the nonparenchymal fraction (Table 1) may just reflect the results being shown as the average of two experiments (± range). It may be that, in these fractions, as reported in other tissues and species, PK 11195 binding exceeds Ro5 4864 possibly due to preferential sensitivity of Ro5 4864 binding sites to inactivation or due to additional PK 11195 binding sites. This small difference has not been further examined in the present study.

Isopycnic density gradient centrifugation of liver 'mitochondrial' fractions resulted in a single peak of PBBS ligand binding in both hepatocyte and nonparenchymal cells (Fig. 1). In the case of the hepatocytes, the peak of [^{3}H]PK 11195 binding was coincident with the activity profiles of both succinate dehydrogenase and MAO_A, markers for the mitochondrial inner and outer membranes, respectively, with a shoulder at a lower density (Fig. 1A). However, the peak of binding of both [^{3}H]Ro5 4864 and [^{3}H]PK 11195 in the gradient fractions from nonhepatocyte cells did not coincide with the mitochondrial markers, but was present at a lower density (Fig. 1B), showing that, in nonparenchymal cells, the receptor resides on a membrane which is not mitochondrial.

Using subcellular fractionation, density gradient centrifugation, density perturbation, and transmission microscopy, we have previously described a nonmitochondrial form of the receptor from rat whole liver that was suggested to be plasma membrane in origin [7]. Immunocytochemical studies from our laboratory have previously shown a high density of receptor in biliary epithelial cells [5]. These separate locations for the receptor in two different types of liver cell, thus, extend our previous observations and demonstrate that, in rat liver, two populations of the receptor exist—a nonmitochondrial, possibly plasma membrane PBBS in nonhepatocyte cells and a mitochondrial outer membrane PBBS in hepatocytes. This nonmitochondrial PBBS, although located in a fraction containing the biliary epithelial cells, would appear not to be on the apical portion of these cells. The activity of γ-glutamyl transferase, a marker for the apical face of the polarised biliary epithelial cell [34], was found at a low density near the top of the sucrose gradient in the case of nonparenchymal 'mitochondrial' fractions (Fig. 1B) and did not enter the gradient in the case of the hepatocyte 'mitochondrial' fractions (Fig. 1A). The specific activity of this enzyme was much higher in the nonparenchymal fractions, which is consistent with a report that 80–90% of the activity in rat liver is in the biliary tract [36].

The finding of a minor proportion of PBBS binding from hepatocyte fractions at the lower density would be consistent with some minor contamination of collagenase-derived hepatocyte preparations with nonparenchymal cells

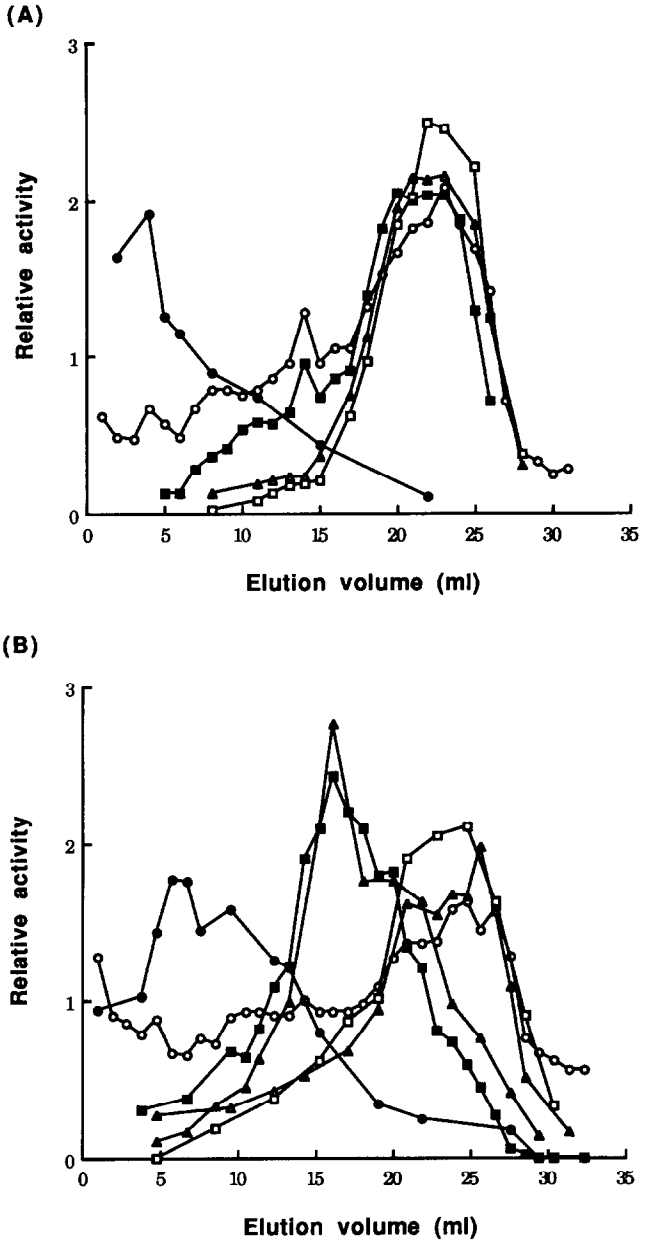

FIG. 1. Sucrose density-gradient centrifugation of 'mitochondrial' fractions. Mitochondrial fractions (3.5 mL, 18 mg protein) prepared from hepatocytes (A) and nonparenchymal cells (B) were layered on top of a continuous sucrose density gradient (1.0–1.6 M) and centrifuged at 97,000 g for 1 hr. Fractions (1 mL) were assayed for protein (○), succinate dehydrogenase activity (□), monoamine oxidase A activity (△), γ-glutamyl transferase activity (●), [^{3}H]PK11195 binding (■), and [^{3}H]Ro5 4864 binding (▲) as described in Materials and Methods. The relative activities (total activity in each fraction divided by the total activity that fraction would have contained if the activity was distributed homogeneously throughout the gradient) were plotted against elution volume (mL) (from the top of the gradient). Each point is the mean of 2 or 3 replicates and the experiment was carried out twice with identical results.

[37]. The lack of any PBBS at the density of the mitochondrial fraction in the nonparenchymal fractions shows that these are essentially free of hepatocytes.

It is difficult to perform relative recovery studies for the preparation of 'mitochondrial' fractions from liver parenchymal and nonparenchymal cells to estimate the relative amounts of liver PBBS in each cell type. Based on specific activities of binding (Table 1), and assuming equal amounts of mitochondria per wet weight of each cell type, equal homogenisation efficiencies, and mitochondrial recoveries, these results suggest that rat liver contains approximately equal amounts of PBBS in each cell type. Our previous studies on fractionation of rat liver whole 'mitochondrial' fractions on sucrose density gradients, however, showed variable proportions of both sites varying from 9:1 to 1:9, a result ascribed to variable homogenisation efficiencies for different cell types. Because intrahepatic biliary epithelial cells represent only about 5% of total cells in liver [38, 39], these results together show that a significant proportion of liver PBR resides in the biliary epithelial cells. The low numbers of these cells explains their preferentially greater staining than hepatocytes on immunocytochemistry [5].

Further characterisation of the PBBS in hepatocyte and nonparenchymal fractions was performed with [^{3}H]PK 14105, a nitrophenyl derivative of PK 11195 that is a selective photoaffinity label for the isoquinoline carboxamide binding site [40]. In both fractions, a single polypeptide was identified with an identical molecular mass of ~18kDa (17.8 ± 1.5 and 18.2 ± 2 kDa) for hepatocyte and nonhepatocyte, respectively (Fig. 2).

Immunocytochemistry

Acyl-CoA-binding protein was purified from rat liver (Fig. 3A) and was used to prepare a polyclonal antiserum as described in Materials and Methods. The antiserum reacted with a single band of 10 kDa on Western blots of purified ACBP and soluble fractions of liver, kidney, and adrenals (Fig. 3B). Using immunocytochemistry with this antiserum to rat liver ACBP, in liver ACBP appeared to be present in both the bile duct epithelial cells and hepatocytes (Fig. 3C). Western blotting of the postmitochondrial supernatant, from both hepatocyte and nonhepatocyte fractions, with anti-ACBP antiserum showed the presence of the putative endogenous ligand in both fractions (Fig. 3B). Western blotting of the 'mitochondrial' fractions from hepatocytes and nonparenchymal cells with anti-18kDa-protein antiserum could not be performed due to the low levels of PBBS. However, it is interesting to note that this anti-18kDa-protein antiserum, which had been raised against the rat adrenal mitochondrial form of the receptor, cross-reacted with the rat liver nonmitochondrial form of the receptor [5]. This, again, suggests, together with the similar molecular mass and binding specificities, that the mitochondrial and nonmitochondrial forms of the receptor are structurally similar.

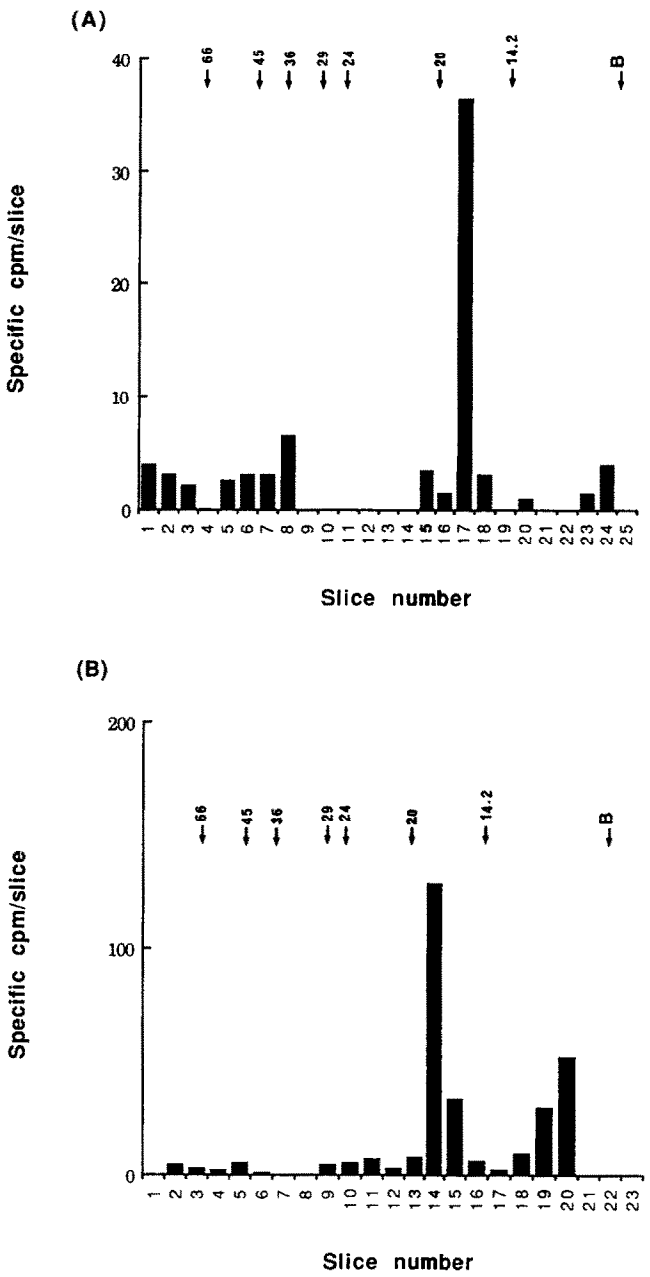

FIG. 2. Gel electrophoresis of mitochondrial fractions photolabelled with [^{3}H]PK 14105. Hepatocyte (A) and nonparenchymal (B) mitochondrial fractions (2 mg protein) were photolabelled with 25 nM [^{3}H]PK 14105 (90 Ci/mmol) in the absence (total labelling) or presence (nonspecific labelling) of 10 μM PK 11195 and aliquots (100 μg protein from hepatocyte fractions, 200 μg from nonparenchymal fractions) were subjected to SDS/PAGE on 15% (w/v) gels. Gel lanes were cut into 2 mm slices, solubilised in 30% hydrogen peroxide (w/w) at 37°C for 26 hr and counted for radioactivity. The results are presented as specific labelling (cpm/slice), after subtraction of the background values obtained with labelling in the presence of PK 11195 (<40 cpm/slice in A; <20 cpm/slice in B) from the values obtained in the absence of this ligand. The migration of standard proteins of known molecular mass (in kDa) was determined by Coomassie staining of the relevant gel lane and is shown by arrows. The position of the Bromophenol Blue dye front (B) is also indicated. The experiment was carried out twice with identical results.

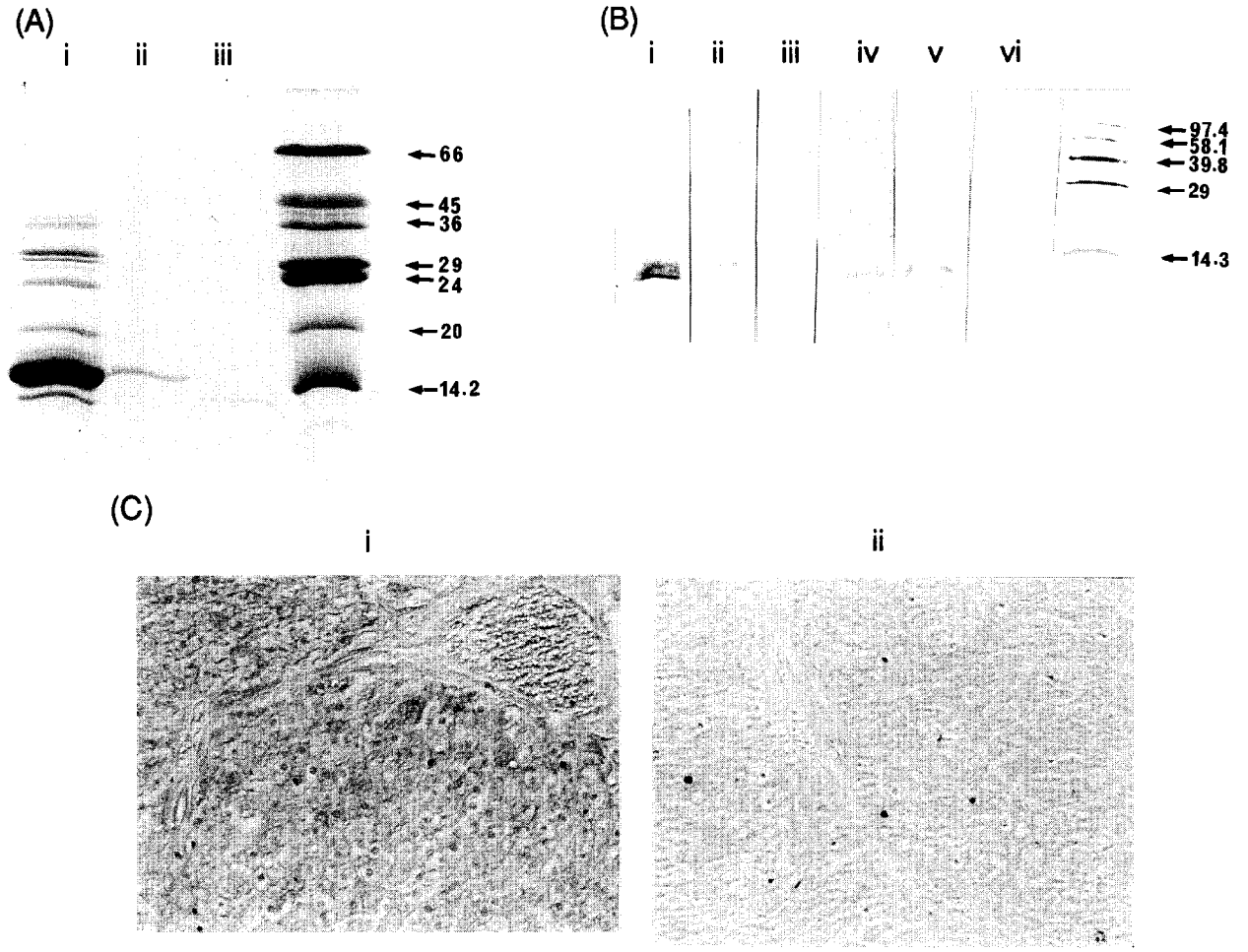

FIG. 3. Immunocytochemical studies with anti-ACBP antiserum. (A): ACBP was purified from rat liver as described in [19]. ACBP-containing fractions were identified by their ability to inhibit specific binding of [^{3}H]PK 11195 to rat adrenal mitochondrial membranes and aliquots (200 μg protein) of the Sephacryl S200 fraction (i), the Sephadex G50 fraction (ii), and the HPLC fraction (iii) were subjected to SDS PAGE on 20% gels and were stained with Coomassie Brilliant Blue. The arrows indicate the migration of standard proteins of known molecular mass (in kDa). (B): Purified ACBP (5 μg) (i) and aliquots (100–200 μg protein) of the soluble fraction from rat liver (ii), adrenal (iii), kidney (iv), hepatocytes (v), and nonparenchymal cells (vi) were subjected to SDS PAGE on 20% gels, the proteins were electrophoretically transferred to nitrocellulose sheets, and the blots were incubated with anti-ACBP serum (1/100 in TTBS). The blots were developed using an ABC Vectastain kit. The arrows indicate the migration of standard proteins of known molecular mass (in kDa). (C): A section of rat liver was immunostained with anti-ACBP antiserum (i) or with preimmune serum (ii), both diluted 1/500, as described in [5]. The magnification is ×40.

To further characterise the properties of liver PBBSs the influence of several compounds on ligand binding was studied. In these studies, whole liver 'mitochondrial' fractions were used to provide sufficient receptor for study.

Effects of Phospholipids

To determine the relationship of PBBS to mitochondrial membrane contact sites, the effects of some phospholipids on the binding properties of liver and adrenal PBBS were examined. Phosphatidyl serine and soybean lipid increased specific ligand binding of both [^{3}H]Ro5 4864 and [^{3}H]PK 11195 to adrenal PBBS (Table 2). Saturation binding curves, in the presence and absence of soybean lipid, showed that the increased ligand binding was due to an increase in B_{max} values (from 61.5 ± 1 pmol/mg protein to 94 ± 2 pmol/mg protein for [^{3}H]Ro5 4864 and from 167 ± 5 pmol/mg protein to 265 ± 7 pmol/mg protein for [^{3}H]PK 11195), with no changes in affinities (results not shown). The specific effects of both soybean lipid and phosphatdiyl serine on membrane-bound receptor binding in adrenals is consistent with previous reports of specific effects of this lipid on protection of the receptor from detergent-induced inactivation and on restoration of detergent-inactivated binding [16, 41]. However, in contrast, liver PBBS binding appeared insensitive to phosphatidyl serine or soybean lipid (Table 2). Liver mitochondrial membranes, albeit in the mouse, have a very low content of phosphatidyl serine [42]. This low level is, possibly, a requirement for a specific mem-

TABLE 2. Effect of phospholipids on binding of [^{3}H]Ro5 4864 and [^{3}H]PK 11195 to adrenal and liver mitochondrial membranes

	Adrenal		Liver	
Phospholipid	Ro5 4864 (%)	PK 11195 (%)	Ro5 4864 (%)	PK 11195 (%)
None	100	100	100	100
Soybean lipid	174 ± 9.7	174 ± 19.7	95 ± 21.7	115 ± 16.1
Phosphatidyl serine	147 ± 4.8	166 ± 20.9	101 ± 22.6	83 ± 18.1
Phosphatidyl inositol	67 ± 6.4	80 ± 9.4	10 ± 3.2	20 ± 2.9
Phosphatidyl ethanolamine	99 ± 10.8	104 ± 12.3	122 ± 29.5	114 ± 13.5
Phosphatidyl choline	119 ± 14.3	137 ± 16.4	91 ± 26.0	70 ± 11.4
Diphosphatidyl glycerol	9.9 ± 1.7	21 ± 1.6	17 ± 2.0	39 ± 3.4

Rat adrenal (20 μg protein) and liver (200 μg protein) mitochondrial membranes were assayed for specific binding of 1.6 nM [^{3}H]Ro5 4864 (38 Ci/mmol) and 1.6 nM [^{3}H]PK 11195 (30 Ci/mmol) in the presence of phospholipids (0.03% w/v). Binding is expressed as a % of that in the absence of added phospholipid and the results are the mean ± SEM of 6 replicates from 2 experiments performed in triplicate.

brane transport process because the intramitochondrial transport of phosphatidyl sereine to its decarboxylase on the liver mitochondrial inner membrane, and the subsequent re-export of phosphatidyl ethanolamine to functionally associated endoplasmic reticulum on the mitochondrial outer membrane, occurs *via* contact sites [43].

Phosphatidyl inositol has previously been reported to protect adrenal PBBS from detergent-induced inactivation [5] and, alternatively, to inhibit rat kidney PBBS ligand binding [15]. In this study, phosphatidyl inositol showed a marked inhibition of binding in rat liver (Table 2), similar to that previously reported for rat kidney [13], and partial inhibition of binding in adrenal (Table 2). The specific effects of phosphatidyl serine and phosphatidyl inositol on PBBS ligand binding suggest that either the PBBS may play a role in some tissues in the synthesis of phospholipids or that these phospholipids may regulate receptor function. Further studies should be designed to elucidate these effects.

Cardiolipin (diphosphatidyl glycerol) has been shown to be an absolute requirement for the translocation function in mitochondria of the adenine nucleotide carrier [44], which has been proposed to form a part of an oligomeric complex with the 18kDa binding protein and porin [16]. Cardiolipin accounts for 20% of the phospholipid content of contact sites and inner mitochondrial membrane, but only 4% of outer membrane in mouse liver [42]. In these studies, cardiolipin had an inhibitory effect on both adrenal and liver PBBS, with the adrenal receptor being slightly more sensitive to inhibition than the liver (Table 2). The increased sensitivity of the [^{3}H]Ro5 4864 binding site, again, is consistent with previous reports of its greater susceptibility to other ligands and reagents [13–15]. It is interesting that cardiolipin stabilization of the ANC does not appear to stabilise PBBS ligand binding—rather, it causes inhibition. This result, then, does not provide evidence for or against interaction of ANC with the PBBS, and is consistent with the conclusion of other researchers that, although ANC may be associated with the receptor, it does not play a role in determining the PBBS pharmacological characteristics [24].

In conclusion, the receptor(s) in liver show(s) differences in sensitivity compared to adrenal (or other tissue) PBBS in phosphatidyl serine, soybean lipid, and phosphatidyl inositol inhibition, possibly indicating differences in receptor conformation(s).

Effects of Modulators of Mitochondrial Respiration

In view of our finding that a large proportion of rat liver PBBS resides in hepatocyte mitochondrial membranes and, because rat liver mitochondria have been well established as suitable for studies of intermembrane contact sites [45], we have studied the influence of contact-site manipulation on rat liver PBBS. The frequency of contact-site formation in state III or phosphorylating liver mitochondria is 4 times greater than in nonphosphorylating, state IV mitochondria [46]. Therefore, uncouplers of respiration, atractyloside (ATR), carbonyl cyanide trifluoromethoxyphenylhydrazone (FCCP), and 2,4-dinitrophenol (DNP), were examined for their effects on the binding of [^{3}H]Ro5 4864 and [^{3}H]PK 11195 to freshly isolated mitochondria from rat adrenals and liver and to adrenal and liver mitochondrial membranes. Although some inhibition of [^{3}H]Ro5 4864 binding in liver was observed with DNP (in mitochondria and membranes) and ATR (mitochondria only), there was no marked stimulation or inhibition either in mitochondrial membranes that had been stored at −20° or in freshly prepared, respiring mitochondria (Table 3). Similarly, conversion of the mitochondria into the state III or phosphorylating condition, (by the addition of respiration buffer containing sodium succinate, ADP, and potassium phosphate) did not have any effect on the binding of either [^{3}H]Ro5 4864 or [^{3}H]PK 11195 to freshly prepared adrenal or liver mitochondria (Table 3). The integrity of the mitochondrial membranes was confirmed by incubating the mitochondria in an oxygen electrode chamber and measuring oxygen consumption under coupled and phosphorylating conditions (results not shown). These observations, that either the uncoupling of respiration or the stimulation of oxidative phosphorylation immediately prior to assay did

TABLE 3. Effect of modulators of respiration on ligand binding to adrenal and liver freshly prepared mitochondria and mitochondrial membranes

	Adrenal		Liver	
	[^{3}H]Ro5 4864 (%)	[^{3}H]PK 11195 (%)	[^{3}H]Ro5 4864 (%)	[^{3}H]PK 11195 (%)
Mitochondria				
No addition	100	100	100	100
+ DNP	87.6 ± 7.5	112.2 ± 2.4	65.9 ± 4.2*	81.4 ± 6.8
+ FCCP	81.2 ± 5.1	112.9 ± 4.6	77.7 ± 13	93.1 ± 6.0
+ ATR	79.7 ± 4.2	112.2 ± 5.2	66.1 ± 9.5*	97.8 ± 7.0
+ Buffer + ADP	96.6 ± 9	108.6 ± 14	95.9 ± 11	91.7 ± 12
Membranes				
No addition	100	100	100	100
+ DNP	89.8 ± 5.1	107.5 ± 6.7	62.9 ± 11*	90.8 ± 9.1
+ FCCP	96.5 ± 1.7	103.1 ± 12	91.2 ± 10	100.2 ± 12
+ ATR	103.0 ± 11	108.0 ± 8.0	90.0 ± 5.2	91.6 ± 9.4

Freshly prepared mitochondria or 'mitochondrial' membranes that had been stored at −20°C from adrenals (20 μg protein) and liver (200 μg protein) were preincubated at 20°C for 10 min with or without 2,4-dinitrophenol (DNP, 30 μM), carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP, 300 nM), or atractyloside (50 μM) before determination of the specific binding of 1.8 nM [^{3}H]Ro5 4864 (40 Ci/mmol) or 1.8 nM [^{3}H]PK 11195 (40 Ci/mmol). Binding to freshly prepared mitochondria was also assayed in the presence of respiration buffer (20 mM Tris/80 mM KCl/5mM $MgCl_2$ pH 7.4) containing sodium succinate (12.5 mM), potassium phosphate (12.5 mM), and ADP (1 mM). All experiments were repeated at least 3 times and assays were carried out in triplicate. The results are presented as mean ± SEM of binding as a % of that in the absence of additions. Significantly different (*$P < 0.05$, ≥4 degrees of freedom) when compared to 100% controls using the Student's *t*-test.

not influence PBBS ligand binding, imply that the formation of contact sites may not be a prerequisite for the active functioning of this receptor in either liver or adrenal tissue. The results are also consistent with the report that, because PBR ligands affect respiration only at high concentrations with no correlation to receptor densities or pharmacology [47], effects on respiration are not mediated through the receptor.

The different locations of the PBBS in two cell types may have functional significance. The mitochondrial receptor has been implicated in the regulation of steroidogenesis [25], but a cell surface plasma membrane location has also recently been described in some other tissues. A PBBS has been described on human erythrocyte membranes [48] and a monoclonal antibody, which had been raised against intact human U937 cells, recognised the PBBS on human neutrophils and was shown to stimulate NADPH-oxidase activation of these cells [49]. Another group has employed the technique of confocal microscopy to demonstrate a cell surface location for a fraction of the receptor in mouse adrenal cortex and in mouse MA10 Leydig cells [10, 24]. In the kidney cells, secreted DBI interacted with the cell surface PBBS to mediate effects on cell growth [24], suggesting multiple roles for this endogenous PBBS ligand.

In the work reported here, we describe for the first time a discrete, but different, location for the receptor in specific cells isolated from the same tissue. In hepatocytes, which contain numerous mitochondria [50], the receptor is mitochondrial whereas, in biliary epithelial cells where mitochondria are sparse [50], the receptor is nonmitochondrial. Molecular biology, protein chemistry, and other studies should aid in establishing structural differences between these two forms. The elucidation of any differences in structure would have important implications for the functional roles of these receptor forms and for their targetting to different membranes. In view of the distribution of both the receptor and DBI-like molecules in two types of liver cells, further studies are necessary to determine the functional roles of the two fractions. A study of the interactions of the endogenous ligand DBI/ACBP with the hepatocyte mitochondrial and biliary epithelial cell nonmitochondrial receptors may elucidate the acyl-CoA-binding function of this protein, in addition to its action as an inhibitor of ligand binding. These two cellular and subcellular locations of the PBBS in liver and the differences in properties compared to other peripheral tissues may be important in view of the multiple roles ascribed to the receptor and to peripheral-type benzodiazepine ligands.

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