

## Structural and Functional Study of Reconstituted Peripheral Benzodiazepine Receptor

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**Recombinant mouse 18 kDa peripheral-type benzodiazepine receptor (PBR) protein was overexpressed in *Escherichia coli* and isolated using a His · Bind metal chelation resin. Recombinant PBR protein was purified with sodium dodecyl sulfate and reincorporated into liposomes using Bio-Beads SM2 as a detergent removing agent. Negative staining of the reconstituted PBR samples, examined by electron microscopy, showed the formation of proteoliposomes. Freeze-fracture of these proteoliposomes revealed the presence of transmembranous particles of an average size of  $3.5 \pm 0.25$  nm, consistent with the presence of a monomeric form of the recombinant PBR protein. The reconstituted protein exhibited the ability to bind both the PBR drug ligand isoquinoline carboxamide PK 11195 and cholesterol with nanomolar affinities. These data suggest that a PBR monomer is the minimal functional unit, binding drug ligands and cholesterol.** © 2001 Academic Press

**Key Words:** PBR; liposomes; cholesterol binding; PK 11195; Bio-Beads; electron microscopy.

The peripheral-type benzodiazepine receptor (PBR) was initially described as a binding site for the benzodiazepine diazepam (1). It was subsequently described as a multimeric complex composed of the 18 kDa receptor protein, the 34 kDa voltage-dependent anion channel (VDAC) protein required for benzodiazepine binding (2) and the 30 kDa adenine nucleotide carrier (3) of an yet unknown function in the complex. Although PBR is present in most tissues examined, it is particularly abundant in steroid producing tissues (4) where it is found in the outer mitochondrial membrane

(5) and the outer-inner mitochondrial membrane contact sites (6). Using high affinity PBR drug ligands, such as the isoquinoline carboxamide PK 11195, it was shown that PBR is involved in the transport of the substrate cholesterol into mitochondria (7), the rate-determining and hormone-dependent step in steroid biosynthesis. Further studies using PBR-mutant steroidogenic cells demonstrated the determining role of this protein in cholesterol transport (8). The binding of cholesterol to PBR was recently demonstrated (9). In addition to its function in steroidogenesis, PBR has been also shown to be implicated in mitochondrial respiration (10), apoptosis (11) and cell proliferation (12) where a PBR-mediated cholesterol compartmentalization might be involved (13).

Initial isolation of the 18 kDa PBR protein from mitochondria was performed using covalently linked drug ligands (14, 15), thus limiting further functional characterization of the partially purified protein in vitro. Subsequent studies using isolated recombinant maltose-binding protein-PBR fusion protein reconstituted into liposomes indicated that, although this fusion protein maintained the ability to bind the high affinity drug ligand PK 11195 (2), it lost its cholesterol binding property (not shown). Thus, at present, there is no information on the structural characteristics of a functional isolated PBR. We report herein the successful isolation and reincorporation into liposomes of a fully functional recombinant mouse PBR protein binding both cholesterol and PK 11195. Structural characterization of the protein indicated that the 18 kDa protein monomer is the minimal functional unit.

### MATERIALS AND METHODS

*Expression and purification of recombinant PBR.* pET15PBR vector was used to transform the BL21(DE3) *Escherichia coli* strain (Novagen, Madison, WI) where the expression of recombinant mouse

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PBR protein was induced by 1 mM isopropyl-1-thiol- $\beta$ -D-galactopyranoside as previously described (16). Cells were harvested and resuspended in binding buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 5 mM imidazole) and sonicated thoroughly. The pellet was collected at 20,000g centrifugation and dissolved in binding buffer containing 1% SDS. The recombinant PBR was purified by the His·Bind metal chelation resin (Novagen, Madison, WI) and stored in binding buffer with 1% SDS as we previously described (9). Protein levels were quantified using the dye-binding assay of Bradford (17) using bovine serum albumin as the standard.

**Reconstitution of recombinant PBR in liposomes.** Solution of the isolated protein was mixed with a suspension of lipids also solubilized in SDS. The resulting micellar protein-lipid-detergent mixture was then transformed into vesicular configuration by detergent extraction with the application of Bio-Beads (18). To analyze the adsorptive capacity of Bio-Beads SM2 (Bio-Rad, Hercules, CA) towards SDS, the concentration of SDS was measured using a specific detergent-sensitive electrode (19). Figure 1A depicts SDS concentration as a function of time for various weights of Bio-Beads applied to the solution. The plateau values of remaining detergent reached in the presence of 20–40 mg beads per ml indicated the adsorptive capacity of the polystyrene beads of about 0.1 mmol or 30 mg of SDS per gram of wet beads. The initial rate of detergent removal is highly dependent upon the number of beads, as clearly illustrated in Fig. 1B. Figure 1C shows that the removal of SDS from either the monomeric state or the micellar configuration remains the same. Depending on the biological nature of phospholipids used in the experiments, either the proteoliposomes, or the pure liposomes, besides isolated protein aggregates, were formed. The highest level of proteoliposome formation was proportionally achieved when PBR was reconstituted in the presence of a mixture of dimyristoyl phosphatyl choline (DMPC) and dimyristoyl phosphatyl ethanolamine (DMPE). Reconstitution with various lipid-to-protein ratios (l/p) led to the conclusion that the proteoliposome formation might be successfully achieved within any lipid to protein ratio higher than 0.25 (w/w).

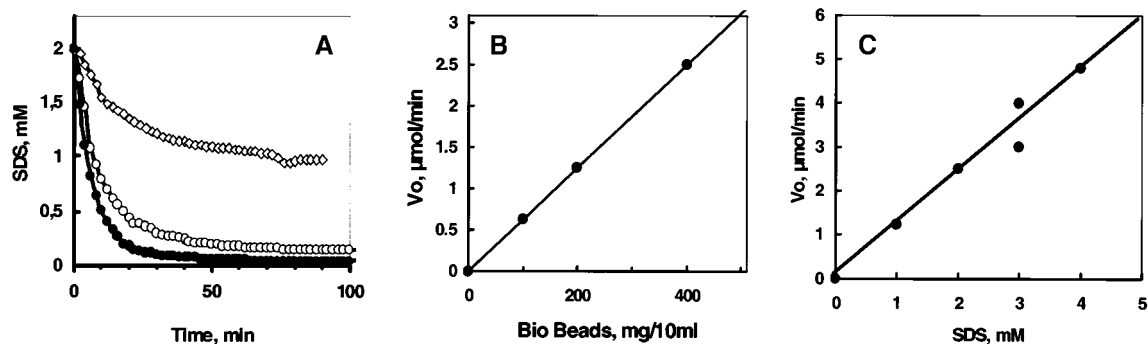
**Radioligand binding assays.** Reconstituted recombinant PBR protein (0.5–2.0  $\mu$ g/ml) in a 4/1 (w/w) lipid (DMPC/DMPE, 9/1) to protein ratio was used for PK 11195 and cholesterol ligand binding studies.  $^3$ H-1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide (PK 11195; sp. act. 83.50 Ci/mmol) binding studies were performed as we previously described (2). In brief,  $^3$ H-PK 11195 binding studies were performed at room temperature, in a final incubation volume of 0.3 ml of phosphate buffered saline (PBS), containing various concentrations of the radioligand with and without 1000-fold excess of unlabelled ligand. After 60 min incubation, assays were stopped by filtration through Whatman GF/B filters (Brandel, Gaithersburg, MD) equilibrated in 0.1% polyethyleneimine and washed with 20 ml ice-cold PBS. Radioactivity trapped on the filters was determined by liquid scintillation counting. [1,2- $^3$ H]-cholesterol (45 Ci/mmol) binding studies were performed as we previously described (16). In brief, proteoliposomes were incubated with increasing concentrations of  $^3$ H-cholesterol in the presence or absence of a 1000-fold excess of unlabelled cholesterol for 60 min at room temperature in a final volume of 0.3 ml. Samples were centrifuged at 10,000g for 30 min, the pellets were washed, resuspended into PBS and bound  $^3$ H-cholesterol was quantified by liquid scintillation spectrometry. Dissociation constants (Kd), the number of binding sites (Bmax) and Hill coefficients (nH) for PK 11195 and cholesterol were determined by Scatchard plot analysis of the generated saturation isotherms using the LIGAND program (KELL v.4.0, Bio-soft Inc., MO) (20).

**Electron microscopy.** Proteoliposomes were placed on glow-discharged carbon-coated grids and subsequently processed for negative staining with 1% uranyl acetate. Samples for freeze-fracture procedures were first coated with platinum under 45°C angle and then with carbon at 90°C angle in a Leica-Reichert apparatus with a total deposit calibrated at circa 20 nm. Biophysical examination of

samples was made in a Philips CM120 electron microscope operated at 120 kV accelerated voltage. The granular diameter of both the negatively stained and freeze-fractured vesicular profiles were determined from images taken at magnifications from 50,000 to 100,000, digitized and morphometrically evaluated. Measurements of the intravesicular particles from freeze-fractured samples were made in accordance with the procedure described by Péranzi *et al.* (21). In brief, measurements of coating shadows (platinum/carbon) over vesicular particles were made directly from the electron microscopic negatives. A Biocom 200 photometric image analysis system and Imagenia software (Imagenia et Instrumentation Biotechnologique, Les Ulis, France) were strictly applied to minimize the possibility of measuring error. The computer program provided isodensitometric contours of all shadows and, in addition, it applied morphometric parameters to the same contours. The population of diameters was a mixture of log normal populations. The parameters of the mixture (i.e., proportions, mean and standard deviation of each component) were estimated by maximum likelihood (22). The concordance of empirical and estimated distributions was assessed by examination of the PP-plot (23). The best concordance is generally obtained when the plot follows the first diagonal.

## RESULTS AND DISCUSSION

Recombinant mouse PBR with a HIS tag at the N-terminus expressed in *E. coli* bacteria retained its ability to bind PBR drug ligands with a pharmacological profile (16) similar to that reported for the native mouse protein (24). Recombinant protein was subsequently purified using the His·Bind metal chelation chromatography to nearly homogeneity in the presence of the ionic detergent SDS (9). The use of SDS resulted in the isolation of a protein which exhibited the ability to bind steroid ligands (9), however it lost the ability to bind the other drug ligands (data not shown). In order to recover a functional PBR protein we reincorporated the recombinant protein into a lipid bilayer. This was achieved through the process of complete detergent removal by the use of Bio-Beads according to the SDS-Bio-Beads specific characteristics described in Fig. 1, the determining factor for a successful reconstitution. Considering these data and previous work on the reconstitution of several other membrane proteins (18), Bio-Beads seems to be, for the first time, a powerful tool for SDS removal and membrane protein reincorporation. Proteoliposomes were examined by electron microscopy. The average value of the vesicular diameter of these proteoliposomes, irrespective of the lipid-to-protein ratio, was estimated at  $200 \pm 100$  nm. The vesicular interior appeared granular with granules evenly distributed throughout the phospholipid bilayer. The average value of the diameter of particles measured from negatively stained samples (Fig. 2A) and the standard deviation were estimated at  $1.5 \pm 0.25$  nm (Fig. 3A). The 18 kDa PBR protein is highly conserved between the four mammalian species cloned (25). Most secondary structure predictions are in agreement with a single PBR molecule consisting of five transmembraneous segments, with C- and N-terminals facing the opposite sides of the bilayer (2,

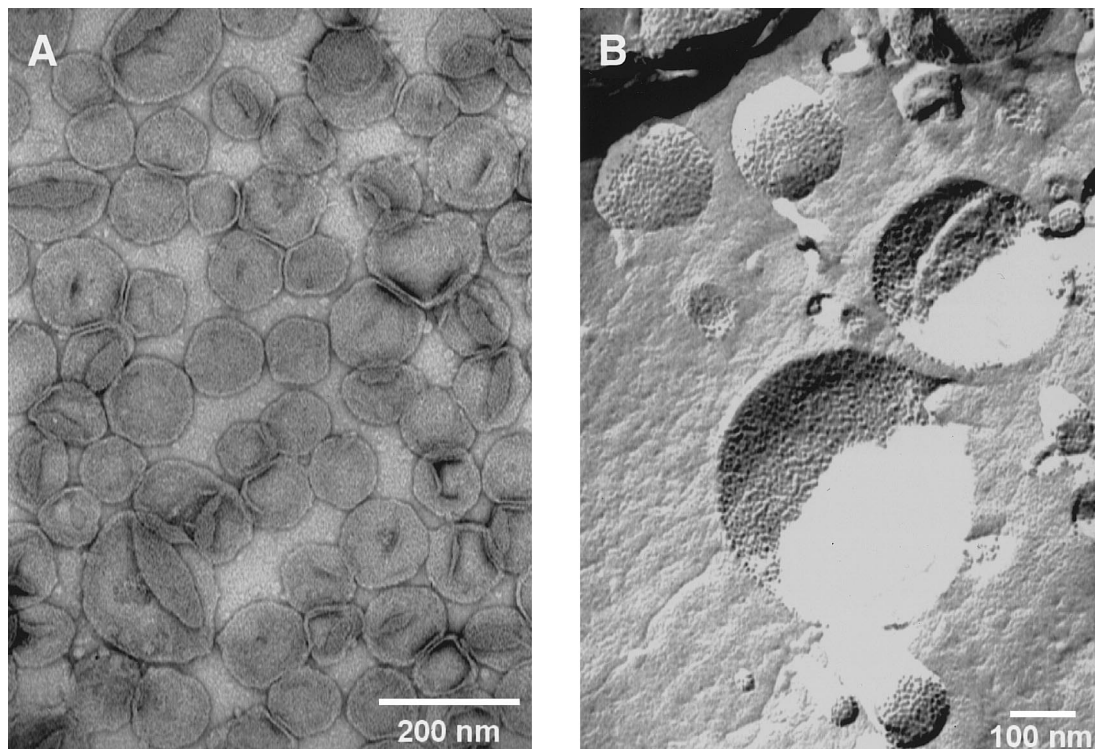


**FIG. 1.** Properties of Bio-Beads SM2 for adsorption of SDS. (A) A 10 ml solution containing 2 mM SDS and 20 mM Tris-HCl (pH 7.5 at 20°C) was treated with 100 (open squares), 200 (closed circles), and 400 (open circles) mgs of Bio-Beads SM2. The change in concentration of SDS was continuously measured with a sensitive-electrode and plotted as function of time for each amount of Bio-Beads used. (B) Initial rates of detergent removal were calculated from data of Fig. 1A and plotted as a function of mg Bio-Beads/ml. (C) initial rate of SDS removal by 400 mg Bio-Beads was plotted as a function of the initial concentration of the detergent.

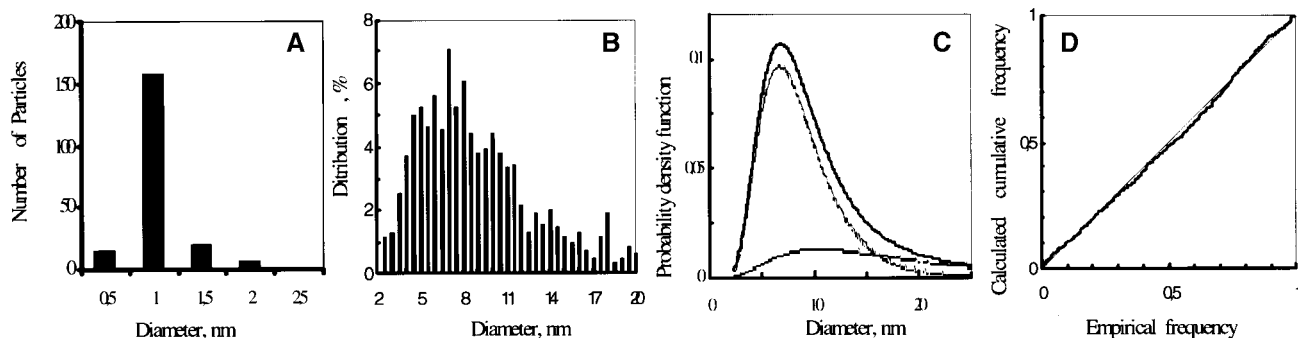
14). These predictions were additionally confirmed by topological analysis using immunodetection of epitopes and specific labeling of cysteine residues (26). The supporting model is composed of short loops on the N-terminal side and longer loops on the C-terminal side. Taking into consideration the results of the 3D construction of the 18 kDa PBR monomer, following the molecular dynamics simulation method (6, 27), it

can be postulated that the particles presently analyzed in the reconstituted proteoliposomes represent merely monomeric conglomerates.

The 1.5 nm diameter appears too small to account for the entire 18 kDa protein consisting of five alpha helices (2, 14). However, it is assumed that in this instance the stain revealed only the extramembranous domains of the protein, which in the case of the PBR represents



**FIG. 2.** Electron microscopy images of negatively stained (A) and freeze-fractured (B) proteoliposomes obtained after reincorporation of recombinant mouse PBR protein. Lipid to protein ratio was at 4.0 (w/w). Detergent removal from the mixture of PBR and DMPC/DMPE (9/1) solubilized in 0.1% SDS in the presence of 500 mM NaCl, 60 mM imidazole, and 20 mM Tris-HCl at pH 7.5 was achieved in 1 h following the application of an appropriate quantity of Bio-Beads SM2. Negative staining and freeze-fracture of the preparations were performed as described under Materials and Methods.



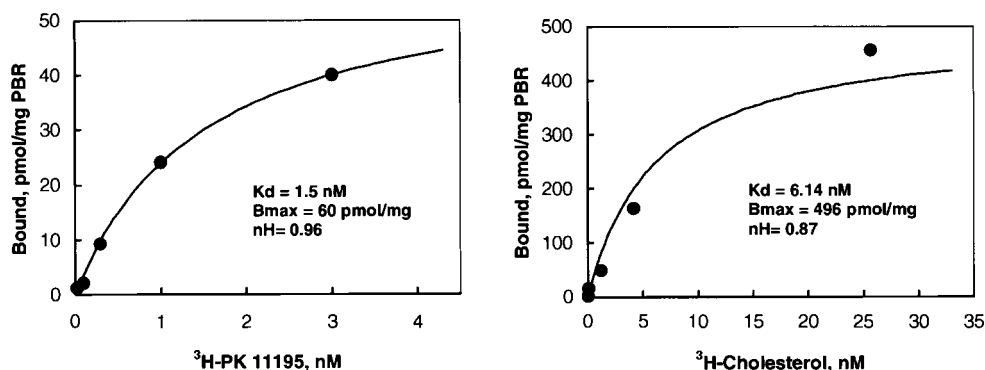
**FIG. 3.** Morphometric analyses of the recombinant PBR protein reconstituted in proteoliposomes. (A) Distribution of diameters of intravesicular particles assessed from negatively stained samples. Number of particles is plotted against the diameters expressed in nm. (B) Distribution of diameters of intravesicular particles assessed from freeze-fractured samples. Number of particles is plotted against the diameters expressed in nm. (C) The calculated distributions are shown as continuous lines. The upper curve is the sum of the two subpopulations. The other two curves are representative of each individual subpopulation. (D) Percentile-percentile-plot of empirical and calculated models pictures out the concordance of empirical (dotted line) and calculated (solid line) distribution.

only 15–20% of the molecular volume. Thus, it is suggested that the obtained value of 1.5 nm merely reflects external, or extramembraneous loops and N- or C-terminus domains of the protein molecule.

Freeze-fracture experiments (Fig. 2B) were performed to characterize the transmembrane domain of the PBR reincorporated in the bilayer. Figure 3B indicates that the diameter of intravesicular particles in this instance shows a great spread of variations, and, for this reason, the particles might be differentiated into the two groups (Figs. 3C and 3D): First, particles varying in size from 2.0 to 20.0 nm; and second, particles varying in size from 6.0 to 30.0 nm. The median width of the first group of particles, representing 93% of the population, was  $8.9 \pm 0.25$  nm. Since these measurements were made directly from the platinum/carbon layers, it was necessary to establish the corrected value of the intra-vesicular particles alone. The latter value was calculated as directly mea-

sured value minus the thickness of the deposited replica (21, 28). Consequently, the corrected average size was derived as  $3.5 \pm 0.25$  nm. The particles classified in the second group, due to the excessive diameter, are suspected to represent non-specific PBR aggregates. The corrected averaged size of 3.5 nm diameter is indeed in good agreement with the concept of five transmembrane helices, thus representing the entire mass of a PBR monomer. In conclusion, these data indicate that we successfully reincorporated the isolated recombinant PBR protein within an artificial bilayer membrane.

We next investigated the functionality of the reconstituted PBR. As noted above, PBR binds with high affinity the isoquinoline carboxamide PK 11195 (4). Figure 4 left shows that the reconstituted protein maintained its ability to bind this drug ligand with high affinity. Pure liposomes, devoid of PBR protein, did not exhibit significant PK 11195 binding (not



**FIG. 4.** Saturation isotherms of  $^3\text{H}$ -PK 11195 and  $^3\text{H}$ -cholesterol binding to reconstituted recombinant PBR. Recombinant mouse PBR protein (0.5–2.0  $\mu\text{g}/\text{ml}$ ) reconstituted in a 4/1 (w/w) lipid (DMPC/DMPE, 9/1) to protein ratio was used. Binding experiments using  $^3\text{H}$ -PK 11195 (left) and  $^3\text{H}$ -cholesterol (right) were performed and analyzed as described under Materials and Methods. Saturation curves are shown and the results of the subsequent Scatchard analyses are indicated in the figures. Results shown are representative of three independent experiments performed in triplicates. The SEM values for  $K_d$ ,  $B_{\text{max}}$ , and  $nH$  were consistently less than 15% of the mean.

shown). In the addition to its ability to bind PK 11195, the reconstituted PBR protein also bound the benzodiazepine Ro5-4864 although with a lower capacity (~20 pmol/mg PBR protein in the presence of 3 nM <sup>3</sup>H-Ro5-4864) compared to PK 11195 (~40 pmol/mg PBR protein in the presence of 3 nM <sup>3</sup>H-PK 11195). Previous studies showed that benzodiazepine binding to PBR required the presence of the 18 kDa PBR, VDAC, and adenine nucleotide carrier proteins (3). Using a recombinant maltose binding protein-PBR fusion protein reconstituted in liposomes we observed that only the isoquinoline carboxamide binding site was expressed (2). Addition of isolated VDAC protein to the liposomes fully reconstituted the benzodiazepine binding capacity of PBR (2). The results presented herein indicate that the reconstituted 18 kDa recombinant PBR protein alone exhibits both the isoquinoline carboxamide and benzodiazepine binding sites in agreement with Joseph-Liauzun *et al.* (28). In our previous studies, the presence of the bulky maltose-binding protein in the amino terminus of PBR may have affected the folding of the PBR protein and blocked the expression of the benzodiazepine binding site, which was recovered in the presence of VDAC. Although in native mouse mitochondria PK 11195 and Ro5-4864 bind to PBR with similar affinity and capacity (24), recombinant mouse PBR expressed in *E. coli* binds with a tenfold higher affinity PK 11195 than Ro5-4864 (16, 29). Although the 18 kDa PBR protein binds both molecules, the difference in the PBR binding capacity for PK 11195 vs Ro5-4864 may be due to the absence of VDAC in the *E. coli* (16) and artificial liposomes used herein.

We previously defined a cholesterol recognition/interaction amino acid consensus in the carboxyl-terminus of PBR (16). Other steroids tested did not interact with this cholesterol binding site (16). We recently examined the PBR-cholesterol interaction by UV crosslinking of the a C17 side-chain containing progestin, promegestone using isolated recombinant mouse PBR in solution (9). Radiolabeled promegestone photoincorporated into recombinant PBR and the CRAC domain of the receptor and this labeling was displaced by cholesterol with an IC<sub>50</sub> of 1 μM, indicating that cholesterol binds to PBR.

Figure 4 right shows that the reconstituted recombinant PBR protein specifically binds cholesterol with high affinity (6 nM) whereas pure liposomes devoid of PBR did not exhibit significant cholesterol binding. It is possible that the removal of the detergent from the protein in solution may have contributed to the unmasking of its ability to bind cholesterol when incorporated into liposomes. In addition, under these conditions PBR may assume a folding similar to the one in its native mitochondrial environment, an important factor for a highly hydrophobic protein to maintain its functional properties. Therefore these results not only confirm the finding that cholesterol binds to PBR but

also demonstrate that cholesterol is a ligand of high affinity for the receptor. The fact that the reconstituted PBR maintained both of its known functional properties suggest that it may be a useful screening tool for the identification of novel high affinity drugs and natural ligands in a high throughput screening system. However, the stoichiometries of maximum binding of both ligands for PBR remains low (10 and 1 mol per 1000 mol of PBR for PK 11195 and cholesterol, respectively). This could be due to several factors involving incomplete reconstitution, bidirectional reincorporation into the bilayer and/or a significant proportion of inactive PBR.

In conclusion, these results demonstrate that the 18 kDa PBR protein is a high affinity binding site for isoquinoline carboxamide and cholesterol. Although the ability of PBR to bind PK 11195 has been previously recognized, this is the first report that PBR binds cholesterol with nanomolar affinity, providing further support to the previous studies showing that PBR is involved in intracellular cholesterol trafficking (7, 13, 16).

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