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In Vitro Reconstitution of a Functional Peripheral-type Benzodiazepine Receptor from Mouse Leydig Tumor Cells

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SUMMARY

The peripheral-type benzodiazepine receptor (PBR) was identified and characterized by its high affinity for two distinct classes of compounds, the benzodiazepines (BZs) and the isoquinolines (IQs). An *M,* 18,000 IQ-binding protein has been identified as the PBR. In this report we isolated and sequenced a 626-base pair cDNA, specifying an open reading frame of 169 amino acid residues with a predicted molecular weight of 18,843, from MA-10 mouse tumor Leydig cells [i.e., mouse peripheral-type benzodiazepine receptor (mPBR)]. Expression of mPBR cDNA in simian virus 40-transformed 3T3 fibroblasts resulted in an increase in the density of both BZ and IQ binding sites. To examine whether the increased drug binding was due to the *M,* 18,000 PBR protein alone or to other constitutively expressed components of the receptor, an *in vitro* system was developed using recombinant mPBR protein. The mPBR cDNA was inserted in the pMAL-c2 vector downstream from the *malE* gene, which encodes maltose-binding protein (MBP). Transfection of the recombinant pMAL-c2 in *Escherichia coli* provided high levels of expression of the MBP-mPBR fusion protein. Purified MBP-mPBR recombinant fusion protein incorporated into liposomes, but not MBP alone, was able to bind IQs but not BZs. Addition of MA-10 mitochondrial extracts to the liposomes resulted in the restoration of BZ binding. The protein responsible for this effect was then purified and identified as the *M,* 34,000 voltage-dependent anion channel protein, which by itself does not express any BZ and IQ binding. These results provide strong evidence that PBR is not a single protein receptor but a multimeric complex in which the IQ binding site is on the *M,* 18,000 subunit and expression of the BZ binding site requires both the *M,* 18,000 and 34,000 voltage-dependent anion channel subunits.

BZs are one of the most prescribed classes of drugs, because of their anxiolytic, anticonvulsant, muscle-relaxant, and hypnotic properties. It has been shown that the pharmacological effects of BZs are mediated via a recognition site on $GABA_A$ receptors in the central nervous system (1). A second BZ binding site has been also identified, present apparently in all tissues examined including the central nervous system (reviewed in Refs. 2 and 3). In distinction to the central $GABA_A$/BZ binding site this second site was named the "peripheral-type BZ binding site" and was primarily localized on mitochondria, although a plasma membrane fraction has been also identified (4). In the past 5 years several studies have reported physiological roles for these binding sites in the regulation of steroid biosynthesis, mitochondrial respiration, cell growth and differentiation, and the immune system and thus the name PBR was proposed (2, 3).

Although the PBR was originally described as a second binding site for the BZ diazepam, which binds with relatively high affinity to both PBR and $GABA_A$ receptors, the use of a series of BZ derivatives and IQs was important in determining its distinct structural specificity (2, 3). The BZ Ro5-4864 binds with high affinity to the PBR and with low affinity to $GABA_A$ receptors (2, 3). Conversely, clonazepam, which binds with high affinity to the $GABA_A$ receptor, exhibits extremely low affinity for the PBR. IQs, which are structurally different from BZs, have much greater selectivity for the PBR, compared with the $GABA_A$ receptor (2, 3, 5). Thus, the successful pharmacological characterization of the PBR was based on its binding capacity for IQs and selected BZs.

In efforts to characterize the PBR, a number of biochemical studies suggested that BZs and IQs bind to different conformational states of the receptor or that the BZ binding site

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ABBREVIATIONS: BZ, benzodiazepine; GABA, γ-aminobutyric acid; PBR, peripheral-type benzodiazepine receptor(s); mPBR, mouse peripheral-type benzodiazepine receptor(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; IQ, isoquinoline; SV40, simian virus 40; PCR, polymerase chain reaction; VDAC, voltage-dependent anion channel; LDAO, lauryl dimethylamine oxide; MBP, maltose-binding protein; IPTG, isopropyl-β-thiogalactopyranoside; Ro5-4864, 4′-chlorodiazepam; PK 11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide; PK 14105, 1-(2-fluoro-5-nitrophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide; PK 1406(7/8), (−/+)-*N*,*N*-diethyl-2-methyl-3-[4-(2-phenyl)quinolinyl]propanamide.

partially overlaps and/or is allosterically coupled to the IQ binding domain (6, 7). Moreover, species specificity of BZ and IQ binding has been reported. A low nanomolar affinity for IQs appears to be conserved among all species, whereas the affinity for BZs is highly variable; rodent PBR exhibits the highest affinity for BZs (2, 3). Furthermore, initial BZ binding studies in mammalian and nonmammalian vertebrates suggested that only higher vertebrates possessed PBR (2, 3). However, binding studies performed with IQs also identified PBR in lower vertebrates (2, 3). Thus, these studies also support the suggestion that two distinct binding domains exist, one for BZs and one for IQs, with the BZ domain being weakly conserved among species.

Photolabeling of PBR, using the radiolabeled IQ PK 14105, identified a protein of M_r 18,000 in a variety of tissues and cells (2, 3, 8). The photolabeled protein was purified (9–11), although the pharmacological profile of the purified protein was not determined, and the corresponding cDNA was cloned from rats (12), humans (13, 14), and cattle (15). Expression studies of the cloned M_r 18,000 PBR cDNA in mammalian cell lines demonstrated that this protein contains the binding domains for PBR ligands (12, 14, 15), because expression of the PBR cDNA resulted in increased numbers of both IQ and BZ binding sites. These results indicated that the determinants for both IQs and BZs were present in the transiently expressed M_r 18,000 protein. However, species-specific differences in the characteristics of the expressed BZ binding were also reported (14, 15) and may reflect differences in the primary amino acid sequences of the M_r 18,000 PBR. Alternatively, the differences observed in BZ binding may be due to the presence of a protein associated with the M_r 18,000 PBR that would alter its structural conformation, resulting in changes of PBR binding characteristics. This possibility was further substantiated by the observation that the cells used for the transfection assays constitutively expressed PBR and thus the putative PBR-associated protein that might determine the structural and functional PBR microenvironment. Moreover, there is biochemical evidence suggesting that the M_r 18,000 PBR may be associated with other proteins. Proteins of M_r 30,000–35,000 can also be photolabeled, nonspecifically, with [^{3}H]PK 14105 and irreversible BZs (10, 16). Furthermore, radiation-inactivation experiments revealed the presence of a M_r 34,000 BZ-binding protein (17) and a M_r 23,000 IQ-binding protein (18). More recently, McEnery *et al.* (19) presented evidence indicating that the M_r 18,000 mitochondrial PBR co-purified with two proteins, of M_r 32,000 and 30,000. These proteins were identified as the VDAC and the adenine nucleotide carrier, respectively (19).

Thus, despite the extensive pharmacological and biochemical characterization of PBR, there is no information on the IQ and BZ binding sites on the receptor. An expression system devoid of PBR or an *in vitro* reconstitution system for PBR should provide the ideal model to answer the questions raised regarding the capacity of the M_r 18,000 PBR protein to bind IQs and BZs and the potential role of PBR-associated proteins. The MA-10 mouse Leydig cell tumor line was chosen for our studies because it was previously proven to be useful in delineating the role of the PBR in mediating the translocation of cholesterol from the outer to the inner mitochondrial membrane, thus regulating steroid biosynthesis (2, 20).

Experimental Procedures

Cells. MA-10 cells were grown in modified Waymouth's MB752/1 medium containing 15% horse serum, as described previously (21). SV40-transformed mouse fibroblastic 3T3 cells (22) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum.

Construction of MA-10 cell cDNA library. Total RNA was extracted from exponentially growing MA-10 cells using the RNAzol B method (Tel-Test, Inc., Friendwood, TX), as described by Chomczynski and Sacchi (23). Poly(A)$^+$ RNA was purified from total RNA using oligo(dT)-cellulose chromatography. The construction of the cDNA library was performed using a commercial cDNA synthesis kit from Stratagene (La Jolla, CA). To synthesize cDNA appropriate for directional cloning, 5 μg of MA-10 poly(A)$^+$ RNA were primed with a oligo(dT)$_{18}$ primer having a *Xho*I site in the 3′ end. *Eco*RI linkers were ligated onto the double-stranded cDNA and the modified *Eco*RI/*Xho*I cDNAs were then ligated into λUni-ZAP arms. The ligated DNA was packaged *in vivo*, using the commercial Gigapack II packaging extract from Stratagene. The primary cDNA library contained 0.5×10^6 plaque-forming units and was amplified to a titer of 3.0×10^9 plaque-forming units/ml.

Screening of the Uni-ZAP MA-10 cDNA library by symmetric PCR. PCR (24) was used to screen the MA-10 cDNA library for the presence of PBR cDNA. PCR was performed using degenerate oligonucleotide primers designed on the basis of homology between the rat, bovine, and human PBR cDNA sequences (12–15) downstream and upstream of the initiation (5′ noncoding primer) and termination (3′ noncoding primer) sites, respectively. The primers, a 32-mer (5′-TCGAATTCGAKCTYYCCWGAACAGCAGYTGCA-3′) and a 30-mer (5′-TACTCGAGCAGGCCCARTGGTCATGAAAGC-3′), were synthesized by Bio-Synthesis, Inc. (Lewisville, TX) and purified on a polyacrylamide-urea gel.

Each reaction mixture contained a library aliquot in SM (100 mM NaCl, 17 mM $MgSO_4$, 1M-Tris·HCl, pH 7.5, 0.1% gelatin) buffer (1.5×10^7 phages), an equimolar ratio of primers (3 pmol), 200 μM levels of each deoxynucleotide triphosphate, and 2.5 units of Hot Tub DNA polymerase (Amersham, Arlington Heights, IL), in a final volume of 100 μl. The samples were denaturated at 94° for 5 min and subjected to 30 amplification thermal cycles (80-sec denaturation at 94°, 2-min annealing at 45°, and 90-sec extension at 72°), followed by a 7-min final extension at 72°, in a Cetus-Perkins thermocycler. PCR products were purified on Prep-A-gene matrix (Bio-Rad, Hercules, CA).

Single-stranded PBR cDNA amplification by asymmetric PCR. Double-stranded PCR products were concentrated with a Centricon-100 microconcentrator (Amicon Division, W.R. Grace, Danvers, MA). The retentate was electrophoresed and the expected 626-base pair fragment was excised and electroeluted using Spectra/Por membranes with a molecular weight cut-off of 12,000–14,000 (Spectrum, Los Angeles, CA). The eluate was recovered and concentrated as described above.

Single-stranded PBR cDNA was generated using aliquots of purified double-stranded DNA in a 100-μl PCR containing 0.2 μM concentrations of either 5′ or 3′ noncoding primer, to generate an excess of single-stranded DNA corresponding to the sense (+) or antisense (−) DNA strand. The single-stranded DNA PCR amplification differed from the double-stranded DNA amplification in that the number of cycles was reduced to 15 and the extension step was increased to 2 min. Single-stranded DNA PCR products were purified using a Centricon-100 concentrator and were further concentrated by lyophilization.

Nucleotide sequencing. Aliquots of single-stranded DNA template and 1 pmol of limiting primer were used for dideoxy chain termination sequencing (25). Fragments were labeled by direct incorporation of 5 μCi of [α-^{35}S]dATP and were sequenced using T7 DNA polymerase (Sequenase version 2.0 DNA sequencing kit; United States Biochemical Corp., Cleveland, OH). Sequences were read from both strands using the 5′ and 3′ noncoding primers, and each sequence was confirmed an average of four times in both directions. Alternatively, internal primers

corresponding to two conserved regions in the coding sequences of the rat, human, and bovine PBR were used to check and complement the MA-10 PBR sequences obtained with the external noncoding primers. These oligonucleotides were a 29-mer (5′-AAAGAATTCYGCCCA-GYCTGGGGKGCTTC-3′) and a 30-mer (5′-TTGAAGCTTGGRT-CCACCAARGCCCAGCCC-3′).

Transient expression of MA-10 PBR cDNA in eukaryotic cells. Sequenced MA-10 PBR cDNA was first cloned in the *Eco*RI/*Xho*I-digested pBluescriptR KS (+/−) phagemid (Stratagene). The plasmid vector pCMV5 (14, 26) (a generous gift from Dr. D. W. Russell, Southwestern University, Dallas, TX) was used to express the MA-10 PBR coding sequence in eukaryotic cells. We generated a 5′ *Eco*RI protruding end/3′ *Xho*I blunt end MA-10 PBR cDNA by filling in with T4 DNA polymerase and subsequent digestion with *Eco*RI. The cDNA containing the entire reading frame was cloned into pCMV5 linearized by *Eco*RI plus *Sma*I double-digestion, downstream of the cytomegalovirus promotor region. The recombinant expression plasmid was amplified in *Escherichia coli* strain XL1-Blue (Stratagene), purified on a Nucleobond-AX cartridge (Macherey-Nagel, Duren, Germany), and further characterized by restriction mapping. SV40-transformed mouse fibroblastic 3T3 cells were transfected at 50% confluence with Lipofectin reagent (GIBCO BRL/Life Technologies, Gaithersburg, MD), as specified by the manufacturer. In brief, for one 60-mm Petri dish 5 μg of DNA were mixed with 50 μg of Lipofectin and incubated with the cells in 3 ml of Opti-MEM medium (GIBCO BRL) for 24 hr at 37°. Then 5 ml of Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum were added to stop the transfection. After an 8-hr incubation at 37°, the medium was removed and cells were grown for an additional 48 hr with fresh medium.

Radioligand binding assays. Cells were scraped from 60-mm culture dishes into 5 ml of phosphate-buffered saline, dispersed by trituration, and centrifuged at 1200 × *g* for 5 min. The cell pellets were resuspended in phosphate-buffered saline. [^{3}H]PK 11195 and [^{3}H]Ro5–4864 binding studies on 50 μg of protein from the cell suspensions were performed as described previously (4, 21). Binding studies on the recombinant receptor were performed using the indicated amounts of protein. Scatchard plots were analyzed by the LIGAND program (27).

Prokaryotic expression. *Eco*RI/*Xho*I-digested MA-10 PBR full-length cDNA was modified to contain *Eco*RI/*Eco*RI sites using an *Xho*I-*Eco*RI adaptor (Stratagene) and was inserted into the *Eco*RI site in the polylinker area downstream from the *malE* gene, which encodes MBP and results in the expression of an MBP fusion protein, in the pMAL-c2 vector (28) (New England Biolabs, Beverly, MA). Expression of the protein is driven by the strong P_{tac} promoter. The vector also carries the *lacI* gene, which codes for the *Lac* repressor. This keeps expression from P_{tac} low in the absence of IPTG induction. Because PBR is not a glycoprotein, it was not expected that bacterial expression of the protein would affect its characteristics.

Purification of the recombinant protein. IPTG (1 M)-induced bacteria were harvested by centrifugation and the cell pellet was frozen at −20° in buffer A (20 mM Tris·HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM sodium azide). The cells were then sonicated, homogenized in a glass-glass Dounce homogenizer, and extracted for 2 hr at 4° with 0.25% digitonin in 50 mM Tris·HCl, pH 7.4,. After centrifugation at 14,000 × *g* for 60 min the supernatant was collected, diluted 10-fold with buffer A, and loaded onto a 2.5- × 10-cm amylose resin (New England Biolabs) column equilibrated in buffer A. The column was washed with buffer A and bound proteins were eluted with buffer A containing 10 mM maltose. Collected fractions were concentrated with a Centricon-10 concentrator (molecular weight cutoff, 10,000) and the fusion protein MBP-mPBR was identified by SDS-PAGE, followed by immunoblotting using antibodies against MBP (New England Biolabs) and PBR (4, 13). Fractions containing the fusion protein were pooled and digested with factor Xa (at a concentration of 1%, w/w, of the amount of fusion protein).

SDS-PAGE and immunoblot analysis. The induction of PBR in *E. coli* and its purification were monitored by SDS-PAGE followed by immunoblot analysis using anti-PBR antiserum (4, 13), anti-MBP antiserum (New England Biolabs), or normal rabbit serum. At each step an aliquot was reserved for protein estimation and a second aliquot was fractionated by SDS-PAGE on a 10 or 15% acrylamide gel (29). Fractionated proteins were electrotransferred onto nitrocellulose membranes (0.45 μm) and the nitrocellulose membranes were subjected to immunoblot analysis using anti-PBR (1/1000) or anti-MBP (1/10,000) antiserum and goat IgG-horseradish peroxidase, with 4-chloro-1-naphthol as color reagent and hydrogen peroxide as substrate (30).

VDAC purification. MA-10 mitochondrial VDAC was solubilized and purified as described by De Pinto *et al.* (31), using the detergent LDAO and hydroxyapatite/Celite column chromatography. VDAC was identified by immunoblot analysis using an anti-VDAC antiserum (19) kindly donated by Drs. M. W. McEnery and S. H. Snyder (The John Hopkins University, Baltimore, MD). In brief, MA-10 mitochondria, prepared as described previously (20), were solubilized with LDAO (2%, w/v) for 30 min in 10 mM Tris·HCl, pH 7.0, 1 mM EDTA, and centrifuged at 17,000 × *g* for 30 min. The supernatant was applied to a prepacked dry hydroxyapatite/Celite (2:1) column. The column was washed with 5 volumes of solubilization buffer and VDAC was eluted with 5 mM potassium phosphate, pH 7.0, supplemented with 50 mM KCl, in solubilization buffer. The elution was then continued with solubilization buffer containing 50 mM potassium phosphate, supplemented with 500 mM KCl. Fractions were collected, and proteins were concentrated/dialyzed in Centricon-10 concentrators and used in reconstitution experiments. SDS-PAGE was performed with acetone-precipitated proteins, and proteins were visualized by silver staining (32). [^{3}H]PK 11195 and [^{3}H]Ro5–4864 binding was performed with all fractions.

Protein measurement. Microgram levels of protein were measured by the method of Bradford (33), using BSA as a standard. Nanogram levels of protein were measured using the colloidal gold assay of Ciesolka and Gabius (34), with BSA as a standard.

Liposome preparation. Liposomes were prepared as described by New (35). In brief, a chloroform solution (20 mg/ml) of different phospholipids and/or lipids was dried under nitrogen. The ratio of the lipids used was based on the published lipid composition of steroidogenic cell outer mitochondrial membrane (36), i.e., 43% phosphatidylcholine, 30% phosphatidylethanolamine, 11% phosphatidylinositol, 8% cholesterol, 5% phosphatidylserine, 4% other. The lipids were then reconstituted in 1 ml of chloroform and dried slowly under nitrogen in a manner to produce a thin lipid film in the bottom of the tube. Cholate (2%) in liposome buffer (50 mM Tris·HCl, pH 7.0, 0.1 M NaCl, 0.1 mM dithiothreitol) was then added in a 2-ml final volume. The mixture was incubated for 30 min at room temperature, to solubilize the lipids, and was placed on ice for 15 min before the addition of the indicated amounts of PBR protein and/or other proteins/protein extracts. The protein/liposome mixture was incubated for 15 min at 0° and used as described in Results. Radioligand binding assays were performed with 20-μl aliquots of the 2-ml liposome preparations.

Electron microscopy. For electron microscopy, liposome preparations were placed on carbon-coated copper grids and negatively stained with uranyl formate, as described by New (35). Specimens were then examined by transmission electron microscopy (35).

Materials. [*N-methyl*-^{3}H]PK 11195 (specific activity, 86 Ci/mmol), [*N-methyl*-^{3}H]Ro5–4864 (specific activity, 88 Ci/mmol), and EN^{3}HANCE reagent were obtained from Du Pont-New England Nuclear (Wilmington, DE). [α-^{35}S]dATP (specific activity, >1000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). PK 14067 and PK 14068 were the gift of Dr. C. Gueremy (Pharmuka Lab., Groupe Rhône Poulenc Sante, Gennevilliers, France). PK 11195 and Ro5–4864 were purchased from Research Biochemicals Inc. (Natick, MA). LDAO was purchased from Serva (Heidelberg, Germany). Cholesterol, lipids, dicyclohexylcarbodiimide, and BSA (fraction V, 99% pure) were from Sigma Chemical Co. (St. Louis, MO). Hydroxyapatite resin and electrophoresis reagents were from Bio-Rad (Richmond, CA). Alumina matrix was obtained from Aldrich Chemical Co. (Milwaukee,

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                                                    GAGCTTCCCAGAAGAGCAGTTGCAATCACC -   1

ATGCCTGAATCCTGGGTGCCTGCCGTGGGCCTCACTCTGGTGCCCAGCCTGGGGGGCTTCATGGGAGCCTAC -  72
MetProGluSerTrpValProAlaValGlyLeuThrLeuValProSerLeuGlyGlyPheMetGlyAlaTyr -  24

TTTGTACGTGGCGAGGGCCTCCGGTGGTATGCTAGCTTGCAGAAACCCTCTTGGCATCCGCCTCGCTGGACA - 144
PheValArgGlyGluGlyLeuArgTrpTyrAlaSerLeuGlnLysProSerTrpHisProProArgTrpThr -  48

CTGGCTCCCATCTGGGGCACACTGTATTCAGCCATGGGGTATGGCTCCTACATAGTCTGGAAAGAGCTGGGA - 216
LeuAlaProIleTrpGlyThrLeuTyrSerAlaMetGlyTyrGlySerTyrIleValTrpLysGluLeuGly -  72

GGTTTCACAGAGGACGCTATGGTTCCCTTGGGTCTCTACACTGGTCAGCTGGCTCTGAACTGGGCGTGGCCC - 288
GlyPheThrGluAspAlaMetValProLeuGlyLeuTyrThrGlyGlnLeuAlaLeuAsnTrpAlaTrpPro -  96

CCCATCTTCTTTGGTGCCCGGCAGATGGGCTGGGCCTTGGCCGATCTTCTGCTTGTCAGTGGGGTGGCGACT - 360
ProIlePhePheGlyAlaArgGlnMetGlyTrpAlaLeuAlaAspLeuLeuLeuValSerGlyValAlaThr - 120

GCCACAACCCTGGCTTGGCACCGAGTGAGCCCGCCGGCTGCCCGCTTGCTGTACCCTTACCTGGCCTGGCTG - 432
AlaThrThrLeuAlaTrpHisArgValSerProProAlaAlaArgLeuLeuTyrProTyrLeuAlaTrpLeu - 144

GCTTTTGCCACCGTGCTCAACTACTATGTATGGCGCGATAACTCTGGCCGGCGAGGGGGCTCCCGGCTCCCA - 504
AlaPheAlaThrValLeuAsnTyrTyrValTrpArgAspAsnSerGlyArgArgGlyGlySerArgLeuPro - 168

GAGTGAAGGCACCCACCCATCAGGAATGCAGCCCTGCCAGCCAGGCACCATGGGTGGCAGCCCATCATGCTT - 576
GluTer - 169

TCATGACTATTGGGCCTGCT
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Fig. 1. cDNA sequence and deduced amino acid sequence of the *M*, 18,000 mPBR protein. The nucleotide sequence starts at the 5′ end of the sense strand and codes for the complete open reading frame, beginning with the initiation codon at nucleotide 1 and ending at the termination codon at nucleotide 510. The amino acid sequence was deduced using the universal genetic code.

WI). All other chemicals were of analytical quality and were obtained from various commercial sources.

Results

The MA-10 mouse Leydig cell tumor line appeared to be a model of choice to study mPBR cDNA because the PBR protein is highly expressed in these cells, compared with other systems, and is functionally linked to the regulation of steroidogenesis (2). A cDNA library was constructed in the Uni-ZAP vector using mRNA obtained from MA-10 cells. An *Eco*RI site and a *Xho*I site were added in the 5′ and 3′ ends, respectively, of the synthesized MA-10 cDNAs, to ensure directional insertion into the cloning vector. The library was amplified in SURE *E. coli* cells to a final titer of 3.0×10^9 plaque-forming units/ml. Two degenerate oligonucleotides designed by homology with the known sequences of the rat, bovine, and human PBR cDNAs were used to probe the MA-10 cDNA library for the presence of PBR, using the symmetric PCR amplification method. Analysis of the PCR products by electrophoresis on agarose gels revealed the presence of a single cDNA species, with an estimated size corresponding to the expected 626-base pair fragment contained between the two primers on the rat PBR. The amplified double-stranded DNA was purified and further used in an asymmetric PCR using one of the primers at a time to produce an excess of single-stranded DNA corresponding to the sense or the antisense PBR cDNA. Both strands were sequenced. The amplified cDNA fragments spanned 626 bases and specified an open reading frame of 169 amino acids (Fig. 1).[2] The nucleotide sequence was verified several times using different preparations of double-stranded cDNA amplified by PCR from the cDNA library. It was also further confirmed by sequencing the double-stranded cDNA cloned into pBluescript SK vector. Hydropathy analysis of the mPBR amino acid sequence showed five potential transmembrane domains (data not shown).

We chose SV40-transformed 3T3 cells to transiently express the mPBR. SV40-transformed 3T3 cells are of mouse origin (22) and, because of the presence of the SV40 large T antigen, are favorable hosts for pCMV vectors that contain the SV40 origin of replication (26). Moreover, this cell line was found to have low levels of specific binding sites for PK 11195 and Ro5–4864 (Table 1). Surprisingly, the affinity of these cells for PBR ligands was higher than that found in other cell types (4, 21, 37) and in nontransformed 3T3 cells (4); a dissociation constant of 0.1 nM was found in SV40-transformed cells versus 1 nM in nontransformed 3T3 cells. Transient transfection of these cells with pCMV5-mPBR resulted in a 3.5-fold increase in the

TABLE 1

PBR-binding characteristics of SV40-transformed 3T3 control cells and cells transfected with pCMV5 or pCMV5-mPBR

Cell transfection and PBR ligand binding were performed as described in Experimental Procedures. Values shown are means ± standard deviations of quadruplicate determinations from two independent experiments.

	K_d	B_{max}
	nM	pmol/mg of protein
[^{3}H]Ro5-4864		
Basal	0.11 ± 0.01	0.76 ± 0.05
Transfected with pCMV5	0.12 ± 0.05	0.52 ± 0.11
Transfected with pCMV5-mPBR	0.16 ± 0.06	2.60 ± 0.16
[^{3}H]PK 11195		
Basal	0.09 ± 0.02	1.08 ± 0.05
Transfected with pCMV5	0.10 ± 0.03	0.95 ± 0.16
Transfected with pCMV5-mPBR	0.08 ± 0.03	3.48 ± 0.35

[2] The nucleotide sequence reported in this paper has been submitted to the GenBank databank, with accession number L17306.

binding capacity of the cells for both the IQ PK 11195 and the BZ Ro5–4864, without any significant change in the affinity of the receptor for these ligands (Table 1). Cell transfection with pCMV5 vector alone did not affect the binding characteristics of the receptor (Table 1). Saturation curves for transfected (with pCMV5-mPBR) and nontransfected cells are shown in Fig. 2. These data suggest that expression of the M_r 18,000 receptor protein is responsible for the increase in the binding capacity of the cells for IQs and BZs. However, because these cells contained endogenous PBR and presumably constitutively expressed PBR-associated proteins that may play a role in ligand binding, it is difficult to attribute the observed increases solely to the M_r 18,000 receptor protein.

Because we were unable to identify a mammalian cell line devoid of PK 11195 or Ro5–4864 specific binding that could be used to study drug binding to the transfected M_r 18,000 receptor protein, we used the pMAL-c2 vector to express the M_r 18,000 PBR protein in *E. coli*. No PK 11195 or Ro5–4864 specific binding was found in *E. coli* cells, cell extracts, or cell membranes (data not shown). In this system the protein is expressed as a fusion protein with MBP. Fig. 3, *left*, shows the IPTG induction of a M_r 60,000 chimeric protein composed of MBP (M_r 42,000) and mPBR (M_r 18,000). Because most of the protein was found in the insoluble inclusion bodies, the MBP-mPBR was extracted using digitonin and purified on an amylose resin (Fig. 3, *right, lane 1*). Despite the fact that only a M_r 60,000 fusion protein was induced by IPTG (Fig. 3, *right*) in >10 experiments performed, after purification we obtained two protein species, of M_r 60,000 and M_r 52,000. Immunoblot analysis of the purified proteins using anti-MBP (Fig. 3, *right, lane 3*) and anti-PBR (Fig. 3, *right, lane 5*) antisera demonstrated that the proteins were recognized by both antisera, thus suggesting that the M_r 52,000 protein is a proteolytic product of the M_r 60,000 MBP-mPBR fusion protein or that this protein results from the premature loss of the transcript from the ribosomes. The yield of fusion protein from the affinity purification was approximately 3 mg/liter of culture.

Cleavage of the MBP-mPBR fusion protein was performed using protease factor Xa; its recognition site is located 5′ to the polylinker insertion site of the mPBR cDNA, and cleavage yielded the M_r 18,000 mPBR protein (data not shown). However, we were unable to further isolate native M_r 18,000 mPBR protein using either amylose affinity chromatography, ion exchange chromatography, hydroxyapatite chromatography, or gel filtration chromatography run with or without detergents, probably because of the hydrophobic nature of the receptor. Thus, for our *in vitro* reconstitution studies we used the MBP-mPBR fusion protein.

MBP-mPBR fusion protein was then tested for its capacity to bind IQs and BZs (Table 2) and was found to specifically bind [^{3}H]PK 11195 but not [^{3}H]Ro5–4864. Because the conditions used to examine the binding capacity of the fusion protein did not reflect the receptor microenvironment in the outer mitochondrial membrane, we then included the receptor in artificially made liposomes consisting of a variety of lipids in the ratios found in the outer mitochondrial membrane of steroidogenic cells (36). Liposome formation was confirmed by electron microscopy of negatively stained preparations (data not shown). Under these conditions MBP-mPBR exhibited a higher specific binding for PK 11195 (63%) but still no Ro5–4864 binding. To further improve the receptor microenvironment, a nonrelated protein, BSA, was added and [^{3}H]PK 11195 binding was further increased by 67%. It should be noted that purified recombinant MBP, produced and purified in a manner similar to that used for MBP-mPBR, with or without BSA, did not exhibit any specific binding of either IQs nor BZs. This is an important control because it shows that bacterial proteins, which may be present in the affinity-purified proteins, do not affect PBR ligand binding. Scatchard analysis of [^{3}H]PK 11195 binding to the recombinant MBP-mPBR protein in liposomes, in the presence of BSA, indicated a dissociation constant of 0.68 ± 0.10 nM (mean ± standard deviation from three independent experiments), close to that described in most tissues (21, 37) (Fig. 4, *left*). To confirm the pharmacological integrity of the recombinant receptor, we performed displacement studies of [^{3}H]PK 11195 binding using a pair of quinoline propanamide enantiomers, (−)-PK 14067 and (+)-PK 14068, that differ in their binding affinities for PBR, and we demonstrated stereospecific binding of these receptors (38). Fig. 4, *right*, shows that the recombinant MBP-mPBR fusion protein bound

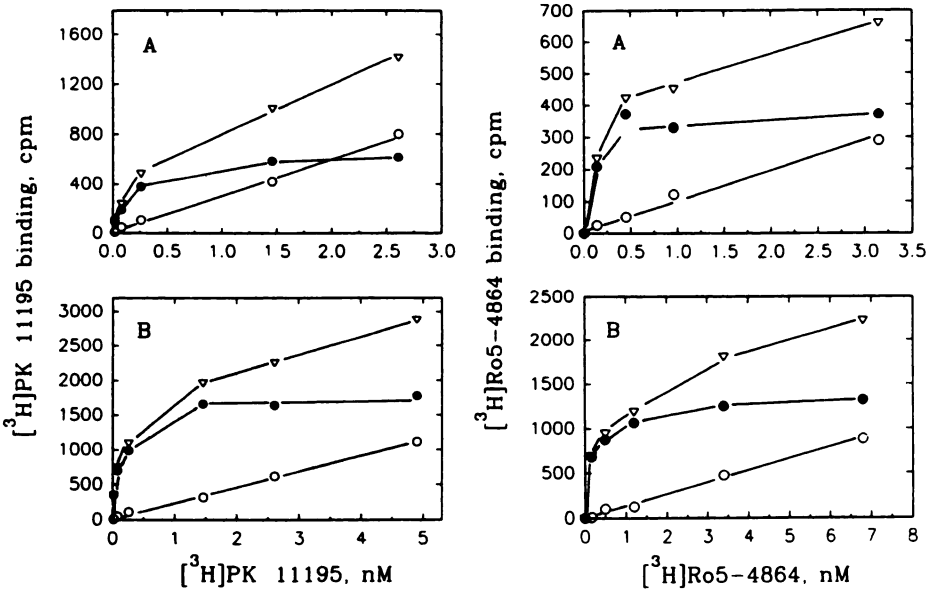

Fig. 2. PBR ligand binding to control (A) and pCMV5-mPBR-transfected (B) SV40-transformed 3T3 cells. *Left*, [^{3}H]PK 11195 binding. *Right*, [^{3}H]Ro5–4864 binding. Binding to cell homogenates was performed as described in Experimental Procedures. ▽, Total binding; ●, specific binding; ○, nonspecific binding. The K_d and B_{max} values obtained are given in Table 1. Nonspecific binding was determined in the presence of a 200-fold excess of unlabeled ligand.

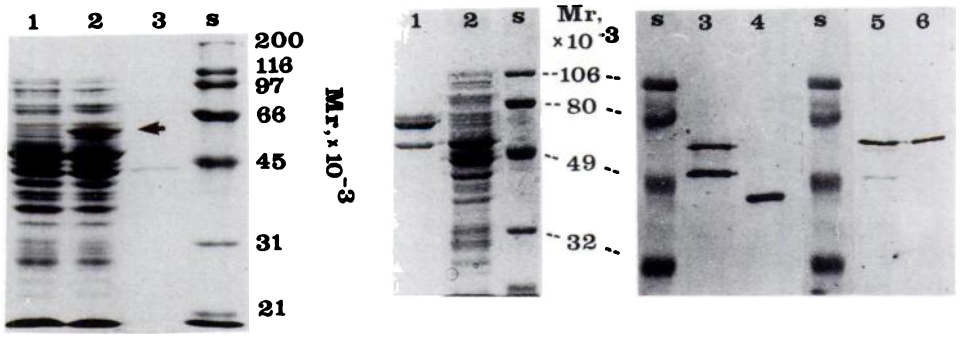

Fig. 3. *Left*, IPTG induction of the MBP-mPBR protein in *E. coli*. Coomassie blue staining of a 10% SDS-PAGE gel containing *E. coli* (TB1) proteins from cells transfected with the pMAL-c2 vector containing the mPBR insert is shown. *Lane 1*, uninduced cells; *lane 2*, cells induced with 0.3 mM IPTG; *lane 3*, purified MBP; *S*, standards. As expected, the molecular weight of the recombinant fusion protein (*arrow*) was 60,000 (M_r 18,000 PBR and M_r 42,000 MBP). *Right*, purification of the MBP-mPBR fusion protein using amylose affinity chromatography. *Lanes 1* and *2*, Coomassie blue staining of a 12% SDS-PAGE gel. *Lane 1*, purified protein eluted from the amylose column with maltose; *lane 2*, unbound proteins from the flow-through fraction from the affinity column; *S*, standards. *Lanes 3-6*, immunoblot analysis of the purified MBP-mPBR proteins (*lanes 3*, *5*, and *6*) and purified MBP (*lane 4*), using anti-MBP (*lanes 3* and *4*) or anti-PBR (*lanes 5* and *6*) antisera; *S*, standards.

TABLE 2

Specific binding of PK 11195 by MBP-mPBR recombinant fusion protein

A mixture of dried lipids, described in Experimental Procedures, was reconstituted with 2 ml of 2% cholate in liposome buffer. The mixture was incubated at room temperature, to solubilize the lipids, and placed on ice, and 10 μg of each protein were added to the lipid mixture. The preparation was incubated for 15 min at 0° and 20-μl aliquots (containing 100 ng of each protein) were used for ligand binding assays. PBR ligand binding was performed as described in Experimental Procedures, in the presence of 1 nM [^{3}H]PK 11195 or 1 nM [^{3}H]Ro5-4864 alone or together with 10 μM unlabeled ligand. Specific binding was >75% of total binding. Values shown are means ± standard deviations from three independent experiments (six determinations).

	Specific binding	
	[^{3}H]PK 11195	[^{3}H]Ro5-4864
	fmol	
Liposomes	ND[a]	ND
Liposomes + MBP-mPBR	9.85 ± 0.68	ND
Liposomes + MBP	ND	ND
Liposomes + MBP-mPBR + BSA	16.47 ± 3.30	ND
Liposomes + MBP + BSA	ND	ND
MBP-mPBR	6.02 ± 0.26	ND

[a] ND, not detectable.

[^{3}H]PK 11195 with high affinity and the IQ enantiomers with the expected apparent affinities of 20 and 300 nM, respectively. Once again, no binding of Ro5–4864, diazepam, or clonazepam was observed, thus indicating that the M_r 18,000 mPBR protein by itself, in the absence of other protein components, exhibits specific binding for IQs only.

In an effort to characterize the PBR protein complex responsible for BZ binding, we initially added total MA-10 mitochondrial extracts to the recombinant MBP-mPBR protein in liposomes. These extracts restored BZ binding to the preparations (data not shown), indicating that, in addition to the M_r 18,000 PBR protein, another component was present and required for BZ binding. We then considered a recent observation made by Snyder and co-workers (19). Those authors reported that the M_r 18,000 PBR protein is associated with the outer mitochondrial membrane VDAC and the inner mitochondrial membrane adenine nucleotide carrier. The data presented by those authors suggested a structural rather than functional association. We used a well established simple protocol to isolate VDAC from MA-10 mitochondria. This procedure uses hydroxyapatite/Celite chromatography of mitochondrial extracts (31). Using this protocol a number of fractions were collected and used to identify immunoreactive VDAC and [^{3}H]PK 11195 and [^{3}H]Ro5–4864 binding. No specific binding was observed in any fractions examined, for either radioligand (data not shown). A silver-stained SDS-PAGE gel of the fractions collected from the hydroxyapatite/Celite column is shown in Fig. 5. A major M_r 34,000 protein, presumably VDAC, is seen in fractions 5–9. However, only in fraction 6 was this M_r 34,000 protein, identified as VDAC by the affinity-purified anti-VDAC antibody used (Fig. 5, *lane IB*), pure enough to be used in our *in vitro* reconstitution studies; an unidentified M_r 70,000 protein was also present in that fraction.

We then examined whether BZ binding to the PBR could be recovered in the presence of VDAC. Thus, we added the purified VDAC (Fig. 5, fraction 6) to liposomes containing the MBP-mPBR fusion protein. Fig. 6, *left*, shows that in the presence of VDAC the Ro5–4864 specific binding to the PBR was recovered. Control experiments were performed by adding to the liposomes (i) purified VDAC (Fig. 5, fraction 6) alone, (ii) purified MBP-mPBR alone, (iii) fraction 2 (Fig. 5), which contains large amounts of the M_r 70,000 contaminant protein, and (iv) fraction 2 (Fig. 5) together with MBP-mPBR. Results obtained from these studies (Fig. 6, *left*) show that these four combinations failed to restore BZ binding, thus demonstrating the specificity of the interaction of VDAC with the MBP-mPBR protein in expressing BZ binding. Scatchard analysis of the [^{3}H]Ro5–4864 binding measured in liposomes containing MBP-mPBR together with VDAC revealed a high affinity binding site with a dissociation constant of 0.90 ± 0.11 nM (mean ± standard deviation from three independent experiments) (Fig. 6, *right*). Fig. 7 further demonstrates the requirement for VDAC for BZ binding. In these studies increasing amounts of VDAC conferred [^{3}H]Ro5–4864 binding to a fixed amount of MBP-mPBR, but no binding was observed with VDAC alone over the same range of protein concentrations. Further pharmacological characterization of the MBP-mPBR/VDAC complex demonstrated a profile (Fig. 8) identical to the well described PBR profile found in a variety of tissues and cells (37).

Discussion

As we discussed in the introduction, numerous studies described and pharmacologically characterized PBR, which bind

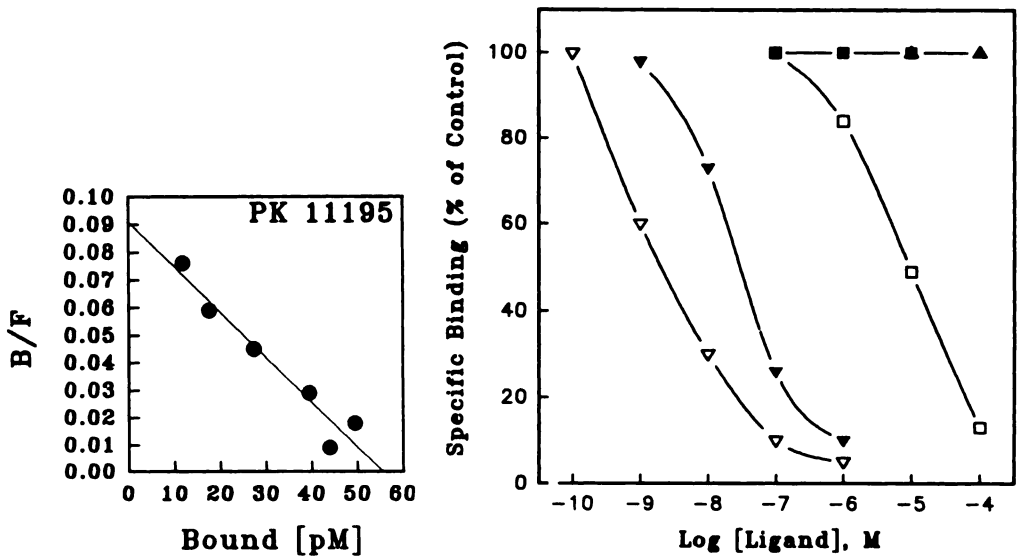

Fig. 4. *Left*, Scatchard analysis of [^{3}H]PK 11195 binding to MBP-mPBR fusion protein reconstituted in liposomes. Scatchard analysis was performed in the concentration range of 0.1–10 nM [^{3}H]PK 11195, using liposomes containing 100 ng of MBP-mPBR and 100 ng of BSA per assay tube, as described in Experimental Procedures and Table 2. Data from a representative experiment are expressed as the means of triplicate assays, where the standard deviation was <10% for all replicates. *Right*, inhibition of [^{3}H]PK 11195 binding to MBP-mPBR fusion protein reconstituted in liposomes. Competition curves for specific binding of 1 nM [^{3}H]PK 11195 are represented relative to the absence of any competitive ligand. In these studies liposomes containing 100 ng of MBP-mPBR and 100 ng of BSA per assay tube were used. One hundred percent binding corresponds to 15.91 fmol of [^{3}H]PK 11195. All data are expressed as the means of triplicate assays, where the standard deviation was <10% for all replicates. Competing ligands used were PK 11195 (▽), (−)-PK 14067 (▼), (+)-PK 14068 (□), Ro5–4864, diazepam, (■) and flunitrazepam, clonazepam (▲). All data are expressed as the means of triplicate assays, where the standard deviation was <15% for all replicates.

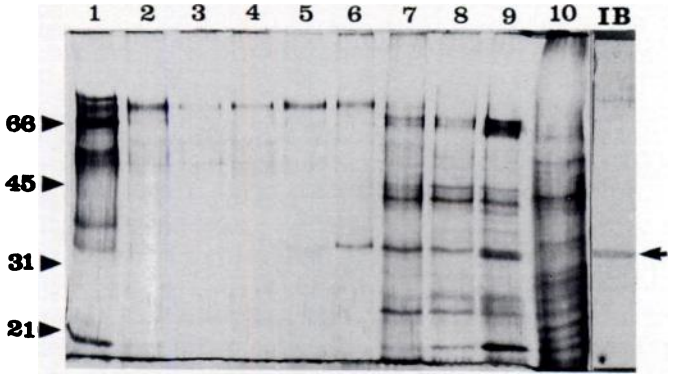

Fig. 5. Purification of MA-10 mitochondrial VDAC by SDS-PAGE of MA-10 mitochondrial proteins purified by one-step hydroxyapatite/Celite column chromatography. Conditions were as described in Experimental Procedures. *Lanes 1-10*, silver staining of mitochondrial proteins eluted from the column on a 10% gel; *lane IB*, immunoblot analysis of fraction 6 with an anti-VDAC antiserum.

IQs and BZs with high affinity, in a variety of tissues and cells (2, 3, 37). The successful detergent solubilization of the mitochondrial PBR, retaining ligand binding (2, 9–11), and the development of a photoaffinity probe specific for PBR, i.e., the IQ derivative known as PK 14105 (8), were the two most important factors in identifying and purifying the IQ binding site of PBR. This photoactivable probe specifically labeled a protein of M_r 18,000 (2, 3, 8, 21). The photolabeled M_r 18,000 protein was subsequently purified (9–11), but the pharmacological characteristics of the native, ligand-free, protein remain unknown. Thus, there is no evidence regarding whether the M_r 18,000 protein confers BZ binding sites, although there is substantial evidence based on (i) chemical and enzymatic perturbation experiments, (ii) species-specific expression, and (iii) differences in the evolutionary appearance of IQ and BZ binding sites (2, 3) that suggests that the PBR has two different conformations or two separate sites on the same protein or that at least two distinct proteins are needed to express the IQ and BZ binding sites.

The PBR has been found to play an important role in a variety of cell functions (2, 3), including the regulation of cholesterol transport from the outer to the inner mitochondrial membrane (2, 20). There cholesterol is metabolized to pregnenolone, the precursor of all steroids synthesized in steroidogenic tissues. This mechanism has been identified in all tissues examined thus far, i.e., testis, adrenal gland, ovary, placenta, and brain (2), although the exact mechanism by which ligand-activated PBR mediates cholesterol transfer remains to be elucidated.

The object of the present study was to identify the structural components of a functional PBR, i.e., a receptor that binds both IQs and BZs. For this purpose the MA-10 mouse Leydig cell tumor line was used. These cells were previously shown to express high affinity IQ and BZ binding sites that were functionally linked to the control of steroid synthesis, the main function of Leydig cells (21, 39). Based on the known rat, bovine, and human nucleotide sequences, sets of primers were synthesized and used to screen by PCR an MA-10 Leydig cell cDNA library. A 626-base pair cDNA encoding a 169-amino acid protein was identified. The encoded protein has a predicted molecular weight of 18,843, which corresponds closely to the M_r 17,000–18,000 IQ-photolabeled protein observed in MA-10 mitochondria (21). Alignment of the deduced amino acid sequence of mPBR with the rat (12), bovine (15), and human (13, 14) sequences revealed 94%, 82%, and 81% homology, respectively. Hydropathy plots used to predict the secondary structure of mPBR revealed five putative transmembrane domains, sim-

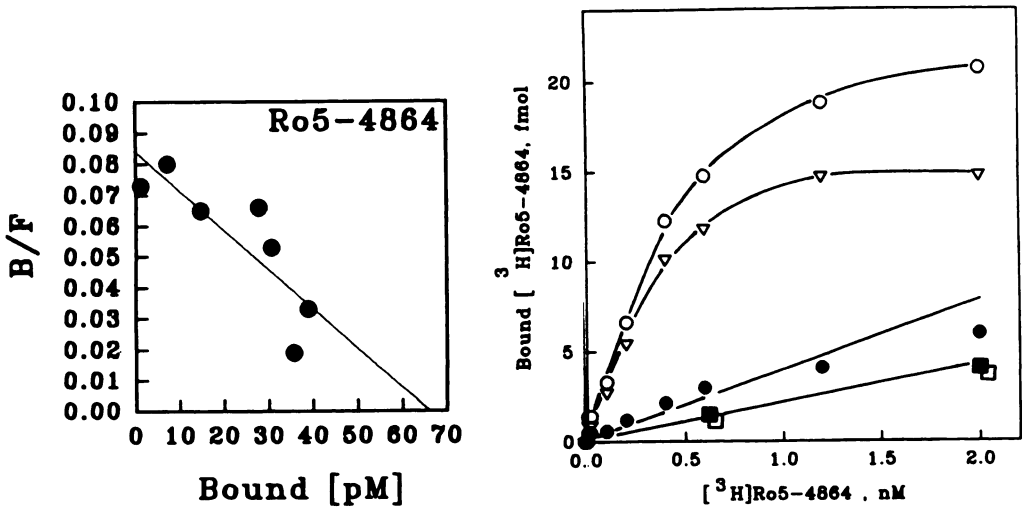

Fig. 6. *Left*, specificity of the interaction of MBP-mPBR with VDAC in conferring BZ binding. A mixture of dried lipids, described in Experimental Procedures, was reconstituted with 2 ml of 2% cholate in liposome buffer. The mixture was incubated at room temperature, to solubilize the lipids, and placed on ice, and 10 μg of BSA together with 2 μg of purified VDAC (Fig. 5, fraction 6), 10 μg of MBP-mPBR, 10 μg of fraction 2 (Fig. 5), 10 μg of MBP-mPBR with 10 μg of fraction 2 (Fig. 5), or 10 μg of MBP-mPBR with 2 μg of purified VDAC (Fig. 5, fraction 6) were added to the lipid mixture. The preparation was incubated for 15 min at 0° and 20-μl aliquots were used for ligand binding assays in the presence of the indicated amounts of [^{3}H]Ro5–4864, with or without a 200-fold excess of unlabeled ligand. Only in the presence of MBP-mPBR together with VDAC was specific binding observed. ○, Total binding; ●, nonspecific binding; ▽, specific binding). *Squares*, total (■) and nonspecific (□) binding obtained under all the other combinations described above. When 0.6 nM ligand was used the values for total and nonspecific binding of [^{3}H]Ro5–4864 (means of triplicate determinations) were as follows: BSA and purified VDAC, 2.66 and 2.97 fmol; BSA and MBP-mPBR, 2.40 and 2.17 fmol; BSA and fraction 2, 2.85 and 3.11 fmol; BSA, MBP-mPBR, and fraction 2, 2.35 and 2.10 fmol, respectively. When 2.0 nM ligand was used the values for total and nonspecific binding of [^{3}H]Ro5–4864 (means of triplicate determinations) were as follows: BSA and purified VDAC, 5.06 and 4.87 fmol; BSA and MBP-mPBR, 5.42 and 5.64 fmol; BSA and fraction 2, 4.96 and 4.73 fmol; BSA, MBP-mPBR, and fraction 2, 3.48 and 4.04 fmol, respectively. *Right*, Scatchard analysis of [^{3}H]Ro5–4864 binding to MBP-mPBR fusion protein reconstituted in liposomes in the presence of VDAC. Scatchard analysis was performed in the concentration range of 0.1–10 nM [^{3}H]Ro5–4864, using liposomes containing 100 ng of MBP-mPBR, 100 ng of BSA, and 20 ng of VDAC per assay tube, as described in Experimental Procedures. Data from a representative experiment are expressed as the means of triplicate assays, where the standard deviation was <15% in all replicates.

ilar to those observed for the cloned rat and bovine PBR (12, 15).

Transfection studies with the mPBR cDNA, using SV40-transformed mouse 3T3 cells as host cells, demonstrated increased expression of high affinity IQ and BZ binding sites, suggesting that the M_r 18,000 protein bears both IQ and BZ binding domains. Similar results were obtained previously using the rat PBR cDNA (12). However, in studies with the bovine and human cDNAs the apparent affinity of the transfected cells for BZs was 100-fold lower (14, 15), suggesting preferential expression of a conserved IQ binding site in these species. In one of these studies Strauss and co-workers (14) demonstrated an important role for the PBR mitochondrial microenvironment in ligand binding and function. Expression studies of human PBR in the yeast *Saccharomyces cerevisiae*, a host cell devoid of IQ and BZ binding, also provided strong evidence that the M_r 18,000 protein contains both the IQ and BZ domains (40). It should be noted, however, that the outer mitochondrial membrane of all organisms so far examined, including *S. cerevisiae*, contains a protein that forms a VDAC when incorporated into planar phospholipid membranes (41). The VDAC has been suggested to be structurally associated with the M_r 18,000 PBR protein (19), and in the present manuscript its functional association with the M_r 18,000 PBR is shown. Thus, use of transfection systems to study IQ and BZ binding by the expressed PBR protein is hampered by the presence of endogenous functional PBR and/or constitutive PBR-associated protein(s)in the cells used for transfection.

To acquire native, ligand-free, purified M_r 18,000 mPBR protein we expressed the corresponding cDNA in *E. coli*. For these studies we chose the pMAL-c2 vector, because in this system the protein of interest is expressed as a fusion protein with MBP. The fusion protein can then be purified through an affinity column, under conditions shown to maintain PBR ligand binding (2, 9–11). As expected, because of the hydrophobic nature of mPBR the MBP-mPBR fusion protein was primarily found in the insoluble inclusion bodies, from which it was extracted using detergent solubilization, maintaining its ligand-binding capacity (2, 9–11). The recombinant MBP-mPBR protein was recognized by antisera raised against MBP and PBR. Interestingly, during the purification procedure partial proteolysis of mPBR occurred, because we consistently observed a second protein of M_r 52,000 that was also recognized by the anti-MBP and anti-PBR antisera. Alternatively, the M_r 52,000 protein may represent a transcipt lost prematurely from the ribosomes. Taking into account the facts that (i) MBP is located upstream of mPBR, (ii) both antisera recognize the M_r 52,000 protein, and (iii) the anti-PBR antiserum was raised against synthetic peptides corresponding to the amino-terminal region of the PBR, we can conclude that the M_r 52,000 protein is MBP (M_r 42,000) and a M_r 10,000 fragment of mPBR. Recombinant fusion protein could be further cleaved by protease factor Xa to yield the M_r 18,000 mPBR, but due to the hydrophobic nature of mPBR we were unable to isolate it from MBP. Thus, we used the recombinant fusion protein MBP-mPBR for the *in vitro* reconstitution studies and used recombinant MBP as a control.

Purified MBP-mPBR fusion protein was able to specifically

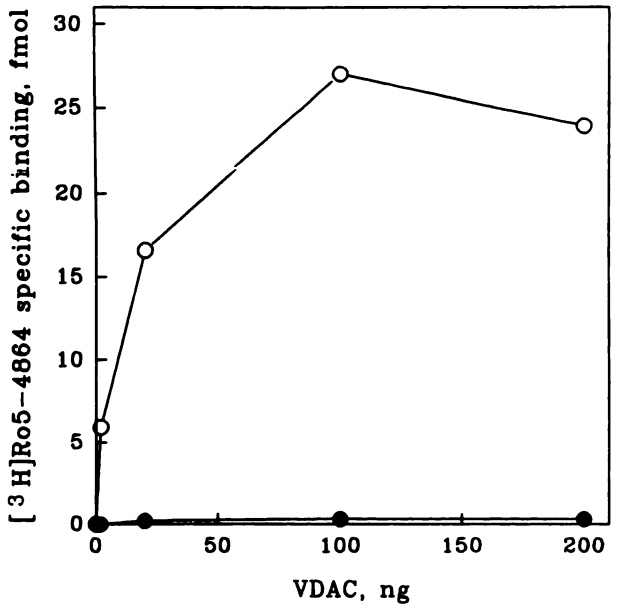

Fig. 7. Effect of increasing concentrations of VDAC on BZ binding. The dried lipid mixture was reconstituted with 2 ml of 2% cholate in liposome buffer, incubated at room temperature to solubilize the lipids, and placed on ice, and 10 μg of BSA together with the indicated amounts (0.2, 2.0, 10.0, and 20.0 μg) of purified VDAC were added with (○) or without (●) 10 μg of MBP-mPBR. The preparation was incubated for 15 min at 0° and 20-μl aliquots were used for ligand binding assays in the presence of 1 nM [^{3}H]Ro5–4864, with or without a 200-fold excess of unlabeled ligand. Data are expressed as the means of triplicate assays, where the standard deviation was <20% for all replicates.

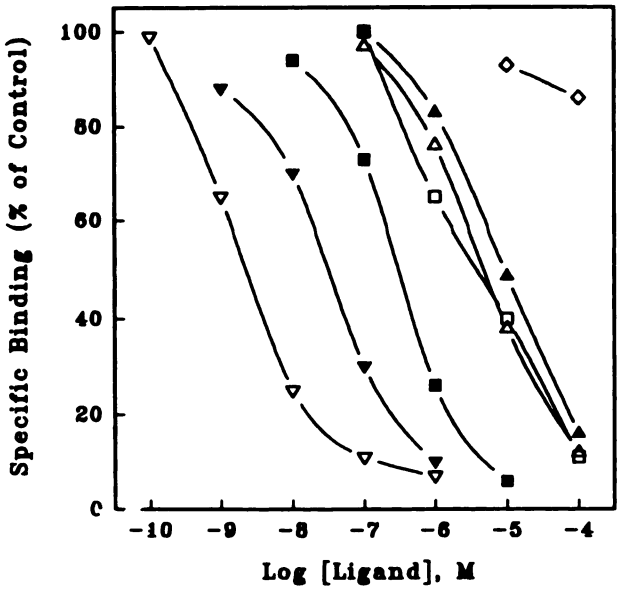

Fig. 8. Inhibition of [^{3}H]PK 11195 binding to MBP-mPBR fusion protein reconstituted in liposomes in the presence of VDAC. Competition curves for specific binding of 1 nM [^{3}H]PK 11195 are represented relative to the absence of any competitive ligand. In these studies liposomes containing 100 ng of MBP-mPBR, 100 ng of BSA, and 2 ng of purified VDAC per assay tube were used. One hundred percent binding corresponds to 18.80 fmol of [^{3}H]Ro5–4864. Competing ligands used were PK 11195 (▽), (−)-PK 14067 (▼), Ro5–4864 (■), (+)-PK 14068 (□), diazepam (△), flunitrazepam (▲), and clonazepam (◇). Data are expressed as the means of triplicate assays, where the standard deviation was <10% for all replicates.

bind the IQ PK 11195. PK 11195 specific binding was dramatically improved by incorporating MBP-mPBR in liposomes consisting of a mixture of lipids in a ratio corresponding to that found in the outer mitochondrial membranes of steroidogenic cells (36). Further addition of BSA to increase the protein/lipid ratio further increased PK 11195 binding. The importance of lipids in supporting PBR ligand binding was first suggested by Anholt *et al.* (42), and more recently it was elegantly demonstrated by Moynagh and Williams (43) in solubilized PBR preparations. Our studies further confirm these observations. Scatchard analysis of PK 11195 binding to recombinant MBP-mPBR, together with displacement studies using the stereospecific IQs, demonstrated the presence of a high affinity IQ binding site on the molecule. Because recombinant MBP (produced and purified as was the MBP-mPBR fusion protein) tested under the same conditions did not confer any IQ binding, we can conclude that the IQ binding was exclusively conferred by the M_r 18,000 mPBR protein. In view of the background information presented above and in the introduction, it was not surprising that the M_r 18,000 protein did not bind BZs. The possibility that MBP may interfere with BZ binding to the MBP-mPBR can be excluded because, as shown below, BZ binding could be restored upon addition of the mitochondrial VDAC to the preparations.

In addition to the M_r 18,000 IQ-labeled PBR protein, a number of proteins of M_r 30,000–35,000 were nonspecifically labeled using irreversible IQs and BZs (2, 3). Among the ligands used to identify these proteins was the BZ flunitrazepam (2, 3). Based on the observation that the M_r 35,000 protein photolabeled with flunitrazepam could also bind radiolabeled dicyclohexylcarbodiimide, a reagent that covalently binds to VDAC, Verma and Snyder (44) advanced the hypothesis that VDAC was part of the PBR. Evidence supporting this hypothesis was presented in experiments using specific reagents that inhibit VDAC channel function, i.e., concanavalin A-Sepharose and Konig's polyanion, and that were able to abolish PBR ligand binding (45). Moreover, we observed that among the PBR ligands tested only flunitrazepam could specifically antagonize, acting via PBR, hormone-stimulated cholesterol transport and steroidogenesis in testicular Leydig and adrenocortical cells (39). More recently, McEnery *et al.* (19) demonstrated that the M_r 18,000 PBR was physically associated with VDAC and the adenine nucleotide carrier, because under a variety of conditions these proteins were co-purified. However, no evidence of a functional association was provided (19). In those studies the authors concluded that IQs bind to the M_r 18,000 PBR protein and BZs bind exclusively to VDAC (19, 45). However, the purified receptor complex, containing the M_r 18,000 protein and VDAC, was identified by photolabeling of the proteins with a variety of ligands. Thus, although those studies provided an initial identification of the receptor complex, no characterization of a functional PBR was shown.

VDAC was purified from MA-10 Leydig cell mitochondria using a well established procedure (31) and the M_r 34,000 VDAC protein was recognized by the anti-VDAC antiserum used. MA-10 Leydig cell VDAC preparations, however, also contained an unidentified M_r 70,000 protein. It should be noted, however, that this M_r 70,000 protein was also present in fractions that were unable to restore BZ binding, where VDAC was absent. Purified VDAC preparations were devoid of any IQ or BZ binding. However, VDAC incorporated together with the MBP-

mPBR fusion protein in liposomes resulted in specific binding of both IQ and BZ ligands. Scatchard analysis and displacement studies identified a high affinity BZ binding site in the MBP-mPBR/VDAC complex. This complex also retained IQ binding. These studies demonstrate that VDAC (i) is functionally associated with the M_r 18,000 PBR, (ii) is part of the BZ binding site in the PBR complex, and (iii) does not bear any IQ- or BZ-binding activity by itself. Although our first observation is in agreement with the proposal of Snyder and co-workers, the other two observations do not agree with that proposal (19, 45). Interestingly, in a recent report mammalian brain VDAC was found to be tightly associated with the central $GABA_A$/BZ receptor protein (46). Thus, it seems that the VDAC primary amino acid sequence and/or the spatial configuration of the molecule have partial recognition sites for BZs. BZ binding, however, is expressed only in the presence of the second protein (M_r 18,000 PBR or M_r 50,000–60,000 GABA receptor), which confers the other part of the recognition site. Alternatively, the BZ recognition site may be exclusively located on either the PBR or VDAC and conformational changes induced by the complementary protein would express the BZ binding site. It is also possible that a certain ratio of M_r 18,000 PBR to VDAC molecules may be a prerequisite for BZ binding to the receptor complex. Although the M_r 18,000 PBR and VDAC are required for IQ and BZ binding, we cannot exclude the possibility that *in vivo* other proteins may be transiently or permanently associated with the M_r 18,000 PBR/VDAC complex, thus modulating IQ and/or BZ binding.

It was previously shown that the M_r 30,000 adenine nucleotide carrier co-purified with the M_r 18,000 PBR protein and VDAC, thus suggesting that its presence is required for BZ binding (19, 45). Because the size of the adenine nucleotide carrier is close to that of VDAC, we also examined the possibility that this protein may be present in the purified VDAC preparations used. Labeling experiments using eosin-5-maleimide, which covalently labels the adenine nucleotide carrier (19), failed to demonstrate its presence in the VDAC preparations used (data not shown). These results suggest that the adenine nucleotide carrier, although it may be associated with the receptor (19, 45), does not play a role in determining the PBR pharmacological characteristics.

VDAC is a large-conductance large-diameter (about 3 nm) ion channel with thin walls formed by a β-sheet structure and is located in the outer mitochondrial membrane, especially in the junctions between outer and inner membranes (contact sites) (47). VDAC forms a slightly anion-selective channel with complex voltage dependence and has been referred to as "mitochondrial porin," by analogy to bacterial porins (41, 47). VDAC is believed to allow transport of metabolites and small molecules between the cytoplasm and the inner mitochondrial membrane (41, 45). In relation to the function of PBR as mediator of intramitochondrial cholesterol transport in steroidogenic cells, it is noteworthy that De Pinto *et al.* (31) demonstrated that the pore-forming protein of the outer mitochondrial membrane (VDAC) of bovine heart contained cholesterol (5 molecules/molecule of VDAC). Therefore, the binding of PBR ligands to the receptor could change the structural interaction of the M_r 18,000 PBR protein with VDAC, subsequently resulting in the release of VDAC-associated cholesterol. Inasmuch as we have recently shown that PBR ligands activate intramitochondrial cholesterol transport and steroid synthesis *in vitro* (2) and that the interaction of the endogenous PBR ligand, the polypeptide "diazepam binding inhibitor," with the receptor mediates the action of hormones on steroid synthesis *in situ* (48), this VDAC-associated cholesterol may represent the first pool to be mobilized and used in steroidogenesis. VDAC has been also shown to be preferentially located in the contact sites, where it may complex with the adenine nucleotide carrier, hexokinase, and creatine kinase (19, 45). Moreover, an increase in the formation of contact sites between the mitochondrial membranes has been observed to occur upon addition of hormones in steroidogenic cells (49). Thus, free cholesterol from the outer mitochondrial membrane would be transferred freely via the contact sites to the inner membrane, where the cytochrome P-450 responsible for side-chain cleavage is located and steroid synthesis begins. Intramitochondrial translocation of phospholipids was recently shown to occur in a similar manner through mitochondrial contact sites (50). This attractive hypothesis, however, remains to be verified.

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