Structure and Function of the Peripheral-Type Benzodiazepine Receptor in Steroidogenic Cells (44215)

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Abstract. **Steroidogenesis depends on the rate of cholesterol transport from intracellular stores to the inner mitochondrial membrane cytochrome P-450 side-chain cleavage enzyme. Using steroidogenic cell submitochondrial fractions, mitochondrial preparations, various cell models, and animal models and with the help of pharmacological, biochemical, morphological, and molecular approaches, we provide evidence that the peripheral-type benzodiazepine receptor mediates the intramitochondrial cholesterol transport and the subsequent adrenal, gonadal, placental, and brain steroid biosynthesis.** [P.S.E.B.M. 1998, Vol 217]

Many tissues of the body have the ability to metabolize steroids. However, few tissues are able to synthesize steroid hormones from the substrate cholesterol (1–4). These tissues are the adrenals, gonads, placenta, and brain. Although both brain glial cells (5) and placenta trophoblasts (6) have all the elements of the steroidogenic machinery and actively synthesize steroids from cholesterol, no hormonal stimuli have been identified to control neurosteroid synthesis and placenta steroidogenesis. However, steroidogenesis in adrenal and gonadal cells has been well characterized, and its regulation by peptide trophic hormones has been extensively studied (1–4). In these tissues, steroidogenesis begins with the conversion of cholesterol to pregnenolone in the inner mitochondrial membrane. Pregnenolone then leaves the mitochondrion to undergo enzymatic transformation in the endoplasmic reticulum that will give rise to the final steroid products. This pathway is essentially regulated by trophic hormones such as ACTH, LH, and FSH, which bind to their specific plasma membrane receptors and activate a stimulatory GTP-binding protein that, in turn, stimulates adenylate cyclase. The stimulation of adenylate cyclase results in an increase in cyclic adenosine monophosphate (cAMP), which is the major second messenger of this system.

The increased cAMP levels trigger three responses: (i) changes in the state of phosphorylation of specific proteins; (ii) induction of protein synthesis; and (iii) stimulation of phospholipid synthesis (1–4). One or all of these cAMP-induced changes will trigger the transport of cholesterol from sites of storage or synthesis to the inner mitochondrial membrane, where C27 side-chain cleavage takes place *via* an enzymatic reaction. This reaction is catalyzed by the C27 side-chain cleavage cytochrome P-450 enzyme (P-450scc), dependent on an electron transport system composed of a ferredoxin and a flavoprotein. Detailed studies have shown that the reaction catalyzed by P-450scc is not the rate-limiting step in the synthesis of steroid hormones, but rather it is the transport of the precursor, cholesterol, from intracellular sources to the inner mitochondrial membrane and the subsequent loading of cholesterol in the P-450scc active site (1–4). This hormone-dependent transport mechanism was shown to be mediated by cAMP and to be localized in the mitochondrion where it regulates the intramitochondrial transport of cholesterol (1–4). The trophic or chronic effect of the peptide hormones results from their effect on the maintenance of appropriate levels of enzymes and other proteins needed for steroidogenesis.

During the last 30 years, a lot of work has been done towards the identification of the mitochondrial cholesterol transport mechanism. Although a number of molecules have

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been proposed as potential candidates mediating this intramitochondrial cholesterol transfer (3, 4), no clear evidence has been presented on the identity of this mechanism. However, during the last decade, a new cholesterol transport mechanism was identified and characterized as mediating the acute stimulation of steroidogenesis by hormones involving the peripheral-type benzodiazepine receptor (PBR) protein (7).

PBR: Pharmacological, Biochemical, and Molecular Characterization

Benzodiazepines are widely used for their anxiolytic, anticonvulsant, and hypnotic actions. It has been well established, that the major pharmacological effects of benzodiazepines are mediated by the γ-aminobutyric acid $GABA_A$ receptors in the central nervous system (CNS) (8, 9). However, in search of specific binding sites for benzodiazepines outside the CNS, another class of binding sites, distinct to $GABA_A$/benzodiazepine receptors, was first observed in the kidney (10) and later determined to be present in apparently all tissues including the CNS (7, 11–13). This class of binding sites is commonly referred to as the peripheral-type benzodiazepine recognition sites or receptors (PBR) due to its initial discovery in peripheral tissues.

In rodent species, PBR bind 4′-chlorodiazepam with high affinity and benzodiazepine diazepam with relatively high affinity whereas clonazepam and flumazenil, which bind with high affinity to $GABA_A$ receptors, exhibit very low affinities for PBR (7, 13). PBR also bind with high affinity the imidazopyridine alpidem and have a low affinity for the imidazopyridine zolpidem, a $GABA_A$ receptor ligand (14). In addition, PBR have high affinity for three classes of compounds, isoquinoline (15), indoleacetamide (16), and pyrrolobenzoxazepine (17) derivatives, which do not bind to the $GABA_A$ receptors (16, 17).

Isoquinolines were the major tool used for the identification and characterization of PBR (7, 12, 13). The first identification of a molecular component associated with PBR was made possible by the development of a photoaffinity probe, the isoquinoline propanamide PK 14105 (18). This probe specifically labeled an 18-kDa protein, which was subsequently purified (19, 20), and the corresponding cDNA was cloned from rat (21), human (22, 23), bovine (24), and murine (25) species. The cDNA sequence of the 18-kDa protein specifies an open reading frame of 169 amino acids, rich in tryptophan residues, with high sequence homology (>80%) across species. Expression studies with the cDNA probes demonstrated that the 18-kDa protein contains the binding domain(s) for PBR ligands although, due to its constitutive expression in all cells used, the presence of other (PBR-associated) proteins important for PBR ligand binding expression cannot be excluded. In support of this hypothesis we should consider that, although high affinity isoquinoline binding is diagnostic for PBR, the affinity of benzodiazepines for PBR is species-specific, varying from high affinity (rodents) to low affinity (bovine) (7, 13). These species differences in benzodiazepine binding also may be due to either structural differences in the 18-kDa protein or differences in the components comprising the PBR complex in the mitochondrial membranes.

No other mammalian protein sharing homology with the 18-kDa protein was identified. However, a 32% amino acid identity (66% when accounting for conserved substitutions) was found with the tryptophan-rich-sensory-protein *tspO* (also called *crtK*), involved in carotenoid biosynthesis in *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* photosynthetic bacteria (26, 27). In these bacteria, where the expression of the genes encoding components of the photosynthetic apparatus is tightly regulated by the oxygen tension and/or light intensity, *tspO* seems to function as an "oxygen" sensor (28). The gene encoding the 18-kDa PBR protein has been isolated and characterized for rat (29) and human (23). In both species the gene contains four exons spanning 10–13 kb, and the locations of the introns are identical.

Subcellular fractionation studies demonstrated that PBR were primarily localized on mitochondria (30–32), and more specifically on the outer mitochondrial membrane (33), although it is likely that they are not exclusive to this organelle, and a plasma membrane location for this receptor was identified (34–36). One of the interesting features of the 18-kDa PBR protein is the absence of a typical mitochondria targeting signal sequence although this protein is targeted to the mitochondria. The amino-terminal sequence of the 18-kDa PBR protein is hydrophobic and resembles a signal peptide, but it is not cleaved when the protein is incorporated in the mitochondrial membrane. In contrast, the carboxy-terminal sequence is hydrophilic, suggesting that it is exposed to the cytoplasmic environment.

We isolated and characterized the 18-kDa PBR cDNA from MA-10 Leydig cells (25). Expression of this cDNA in mammalian cells resulted in an increase in the density of both benzodiazepine and isoquinoline binding sites. In order to examine whether the increased drug binding is due to the 18-kDa PBR protein alone or to other constitutively expressed components of the receptor, an *in vitro* system was developed using recombinant PBR protein (25). Isolated maltose binding protein (MBP)-PBR recombinant fusion protein incorporated into liposomes, formed using lipids found in steroidogenic outer mitochondrial membranes, but not MBP alone, maintained its ability to bind isoquinolines but not benzodiazepines. Addition of mitochondrial extracts in the liposomes resulted in the restoration of benzodiazepine binding. The protein responsible for this effect was then purified and identified as the 34-kDa voltage-dependent anion channel (VDAC) protein, which by itself does not express any drug binding. Interestingly, a number of laboratories have identified a 30- to 35-kDa protein, nonspecifically labeled using irreversible isoquinolines and benzodiazepines, to be associated with PBR (7, 11–13). Among the ligands used to identify this 30- to 35-kDa protein was flunitrazepam. Based on the observation that the

35-kDa protein photolabeled with flunitrazepam could also bind radiolabeled dicyclohexylcarbodiimide, a reagent that covalently binds to VDAC, and that specific reagents that inhibit VDAC function were able to abolish PBR ligand binding, the hypothesis that VDAC was part of PBR was advanced (11). Moreover, we observed that among the PBR ligands tested, only flunitrazepam could specifically antagonize, acting *via* PBR, the hormone-stimulated cholesterol transport and steroidogenesis (27). Furthermore, the observation that the 18-kDa PBR was isolated as a complex with the 34-kDa VDAC and the inner mitochondrial membrane adenine nucleotide carrier (38) suggested that PBR is not a single protein receptor but a multimeric complex.

These studies demonstrated that VDAC is functionally associated to the 18-kDa PBR and is part of the benzodiazepine binding site in PBR. However, benzodiazepine binding will be expressed only in the presence of the 18-kDa PBR protein that confers the other part of the recognition site. This model is also in agreement with the finding that the species difference in benzodiazepine binding may be due to five nonconserved amino acids in the C-terminal end of the 18-kDa PBR protein (39). Although the 18-kDa PBR and VDAC are required for drug binding, we cannot exclude the possibility that *in vivo* other proteins may be transiently or permanently associated with the PBR complex and modulate drug binding in an "allosteric" manner.

VDAC is a large-conductance, large-diameter (about 3 nm) ion channel with thin walls formed by a β-sheet structure and located in the outer mitochondrial membrane, especially in the junctions between outer and inner membranes (contact sites) where it may complex with the adenine nucleotide carrier, hexokinase, and creatine kinase (40). VDAC forms a slightly anion-selective channel with complex voltage dependence and has been referred to as "mitochondrial porin" by analogy to bacterial porins. VDAC is believed to allow transport of metabolites and small molecules between the cytoplasm and the inner mitochondrial membrane (40, 41).

PBR Topography in Mitochondrial Membranes

Native MA-10 Leydig tumor cell mitochondrial preparations were examined by transmission electron and atomic force microscopic procedures to investigate the topography and organization of PBR. Mitochondria were immunolabeled with an anti-PBR antiserum coupled to gold-labeled secondary antibodies. Results obtained indicate that the 18-kDa PBR protein is organized in clusters of 4–6 molecules (42). Moreover, on many occasions, the inter-relationship among the PBR molecules was found to favor the formation of a single pore. Since the 18-kDa PBR protein is functionally associated with the pore-forming 34-kDa VDAC, which is preferentially located in the contact sites of the two mitochondrial membranes, these results suggest that the mitochondrial PBR complex may function as a pore.

We then examined whether the hormone-induced biochemical changes, increased PBR binding, correlated with appropriate morphological changes. A 15-sec treatment with hCG induced the appearance of large clusters varying from 15–25 gold particles, or more, in contrast to the 4–6 particle clusters present in mitochondria from control cells. AFM analysis of these areas further demonstrated the reorganization of the membrane at these mitochondrial membrane sites (43). The specificity of the effect of hCG was determined by treating cells with hCG and the selective inhibitor of cAMP-dependent protein kinase H-89, shown to block the hormone-induced PBR binding and steroid formation. H-89 also blocked the effect of hCG on PBR topography. In addition, flunitrazepam also blocked the effect of hCG on PBR distribution on mitochondrial membranes (43). Thus, it seems that hormones induce the rapid reorganization of mitochondrial membranes favoring the formation of contact sites that may facilitate cholesterol transfer from the outer to the inner mitochondrial membrane. An increase in the formation of contact sites between the mitochondrial membranes has been previously reported (44). Thus, free cholesterol from the outer mitochondrial membrane would transfer freely *via* the contact sites to the inner membrane where the P-450scc is located. It also should be noted that intramitochondrial translocation of phospholipids was recently shown to occur in a similar manner through mitochondrial contact sites (45).

Molecular Modeling of PBR

Based on the known amino acid sequence of the human and mouse 18-kDa PBR protein, a three-dimensional model of this receptor protein was recently developed using molecular dynamics simulations (46, 47). According to this model the five transmembrane domains of PBR were modeled as five α-helices that span one phospholipid bilayer of the outer mitochondrial membrane. This receptor model was then tested as a carrier for a number of molecules, and it was shown that it can accommodate a cholesterol molecule and function as a channel. Thus, it was suggested that the receptor's function is to carry cholesterol molecules from the outer lipid monolayer to the inner lipid monolayer of the outer membrane thus acting as a "shield" hiding the cholesterol from the hydrophobic membrane inner medium. Considering the PBR complex formation at the level of the contact sites, this cholesterol movement could end in the inner mitochondrial membrane. Thus, this theoretical model further supports our experimental data on the role of PBR in intramitochondrial cholesterol transport.

Effect of PBR Drug Ligands on Steroidogenesis

Two important observations indicated that PBR are likely to play a role in steroidogenesis: first, PBR are found primarily on outer mitochondrial membranes; and second, we and others showed that PBR are extremely abundant in steroidogenic cells (7). We then reported that a spectrum of ligands that bind to PBR with affinities ranging from n*M* to m*M* stimulate steroid biosynthesis in various cell systems (48, 49). The relationship between the affinities of these

compounds for PBR and the concentrations of each compound required to stimulate steroidogenesis was examined and showed an excellent correlation, with a coefficient $r = 0.9$, suggesting that these drugs, *via* binding to PBR, stimulate steroidogenesis. However, the stimulatory effect of PBR ligands was not additive to the stimulation by hormones and cAMP (50). Considering the mitochondrial localization of PBR, we then examined the direct effect of PBR ligands on mitochondrial steroid formation. PBR ligands were found to stimulate pregnenolone production by isolated mitochondria (49). This effect was greater with ''cholesterol-loaded'' mitochondria prepared from cells treated with hormone and the protein synthesis inhibitor, cycloheximide (50). This treatment increases the amount of cholesterol present in the outer mitochondrial membrane (2, 4). The stimulatory effect of PBR ligands on intact mitochondria was not observed with mitoplasts (mitochondria devoid of the outer membrane) in agreement with the outer mitochondrial membrane localization of the receptor (49). In these studies we concluded that PBR are implicated in the acute stimulation of adrenocortical and Leydig cell steroidogenesis possibly by mediating the entry, distribution, and/or availability of cholesterol within mitochondria.

In order to identify the exact step in mitochondrial pregnenolone formation activated by PBR ligands we quantified the amount of cholesterol present in the outer and inner mitochondrial membranes before and after treatment with PBR ligands. The results obtained clearly demonstrated that the PBR ligand-induced stimulation of pregnenolone formation was due to PBR-mediated translocation of cholesterol from the outer to the inner mitochondrial membrane (50). However, PBR ligands induced a massive translocation of cholesterol, 10 μg/mg of protein. Considering that only a portion, 10%–20%, of this cholesterol will be used for steroidogenesis and that PBR are present in most tissues examined, these data indicate that PBR-mediated lipid translocation also may be involved in a more general mechanism such as membrane biogenesis. Thus, the abundance of PBR in steroidogenic tissues together with the tissue-specific cholesterol transport make PBR a regulator of this rate-determining process. Studies by different laboratories corroborated these observations (51, 52) and extended them to ovarian granulosa cells (53).

Interestingly, a similar mechanism was shown to regulate steroid synthesis by the two ''nonclassic'' (in terms of the regulatory mechanisms involved) steroidogenic tissues, the placenta trophoblasts (54) and brain glial cells (55).

Initially we examined PBR in brain tissue, primary glial cultures, and C6-2B glioma cells. Subcellular fractionation indicated that the majority of PBR is localized in the mitochondrial fraction, and ligand binding studies, photolabeling, and immunological studies indicated that mitochondria from primary glial cells and C6-2B glioma cells exhibited a high density for the 18-kDa PBR suggesting that, within the CNS, PBR are found primarily in glial cells (32, 56). In addition, the glioma receptor expressed an identical pharmacological profile to adrenocortical and testicular Leydig cell PBR.

We then investigated whether PBR ligands affect pregnenolone formation in C6-2B glial cell mitochondria. At nanomolar concentrations, PK 11195 and Ro5-4864 induced a 2-fold stimulation of mitochondrial steroid production (55). A similar increase was obtained with anxiolytic benzodiazepines that bind to both classes of benzodiazepine recognition sites, whereas clonazepam, a ligand selective for $GABA_A$ receptors, was ineffective at all concentrations tested (55). In these studies, exogenous cholesterol was not supplied to the mitochondria, suggesting that PBR facilitates the transport of cholesterol from the outer mitochondrial membrane to the inner membrane, which is then metabolized by the P-450scc to form pregnenolone. These data were confirmed by others using rat brain mitochondria (57, 58). Moreover, PBR drug ligands were found to stimulate pregnenolone biosynthesis by C6-2B glioma cells in culture (59). In addition, to the *in vitro* and *in situ* studies presented above, PBR drug ligands were found to increase rat forebrain pregnenolone synthesis *in vivo* (60) and to elicit antineophobic and anticonflict actions, presumably *via* their PBR-mediated steroidogenic effect and the subsequent action of the synthesized neurosteroids on the $GABA_A$ receptor. These data demonstrate that PBR is a common mechanism in all steroidogenic tissues.

Recently, a PBR-dependent cholesterol transport mechanism from the outer to the inner mitochondrial membrane was identified in liver mitochondria (61). Cholesterol transport to the liver inner mitochondrial membrane may be required for cholesterol detoxification from the periphery by the sterol-27-hydroxylase (62). Interestingly, the rate of cholesterol uptake and transport from the outer to the inner mitochondrial membrane, in response to PBR ligand activation, was identical (0.9 nmol/mg protein/min) for adrenal (50) and liver (61) mitochondria, suggesting that a similar PBR-mediated cholesterol transport mechanism is operative in both steroidogenic and nonsteroidogenic tissues. Thus, it seems that the regulation of intramitochondrial cholesterol transport may be a general function of PBR.

In addition to its function in cholesterol transport, it was recently reported that benzodiazepines stimulate deoxycorticosterone to aldosterone conversion by adrenal zona glomerulosa cells (52), suggesting that PBR may also mediate deoxycorticosterone transport to the mitochondrial 11β-hydroxylase.

It should be noted that the stimulatory effect of PBR drug ligands is seen using nanomolar concentrations, close to the Kd of the receptor. PBR drug ligands used at high micromolar concentrations inhibit steroidogenesis by competing with endogenous substrates for microsomal and mitochondrial steroidogenic enzymes and by antagonizing the trophic effects of voltage-sensitive calcium channel–dependent stimuli (52). These inhibitory effects are not related to PBR and may represent a pharmacologic/toxicologic action of the drugs or an effect *via* another low

affinity binding site. It this regard, it should be noted that PBR drug ligands at high micromolar concentrations have been reported to affect calcium channel function in a non-specific manner (7).

In addition to the *in vitro* studies presented above, there are some *in vivo* findings that support the role of PBR in steroidogenesis: (i) stress-induced elevations in glucocorticoid plasma levels were attenuated by benzodiazepines (63); (ii) administration of the benzodiazepine diazepam increases plasma testosterone levels in men (64); (iii) high-affinity PBR ligands increase glucocorticoid plasma levels in hypophysectomized rats (65, 66); and (iv) specific PBR ligands block ACTH-induced glucocorticoid production in hypophysectomized animals (66). These studies, not only verify our *in vitro* results, but also demonstrate the crucial *in vivo* role of PBR in the regulation of steroid biosynthesis. In summary, the function of PBR in mitochondrial steroidogenesis seems to be a common element of all steroidogenic tissues in all species examined.

PBR in Hormone-Stimulated Steroidogenesis

Despite the data presented on the effect of PBR ligands on basal steroid synthesis it was still unclear whether PBR participate in the hormone-stimulated steroidogenesis. In search of a PBR drug ligand that may affect hormone-stimulated steroid production, we found that flunitrazepam, a benzodiazepine that binds to PBR with high nanomolar affinity, inhibited hormone and cAMP-stimulated steroidogenesis (37). Radioligand binding studies revealed a single class of binding sites for flunitrazepam, which was verified as being PBR by displacement studies using a series of PBR ligands. Furthermore, this drug caused an inhibition in mitochondrial pregnenolone formation, which was determined to result from a reduction of cholesterol transport to the inner mitochondrial membrane P-450scc. These observations demonstrated that the antagonistic action of flunitrazepam on hormone-stimulated steroidogenesis is mediated through the interaction of this compound with PBR. However, it should be noted that flunitrazepam is also a weak stimulator of basal steroid production suggesting that it acts as a partial agonist of the receptor-mediated steroid synthesis process. In conclusion, these studies suggested that hormone-induced steroidogenesis involves, at least in part, the participation of PBR.

Hormonal Regulation of PBR

We examined whether hCG or cAMP regulates PBR expression in Leydig cells measured by ligand binding and RNA (Northern) blot analysis. Treatment of MA-10 cells from 10 min up to 24 hr with hCG was without any effect on PBR binding or message levels (67). However, addition of hCG to MA-10 cells resulted in a very rapid increase in PBR binding capacity (3-fold increase within 15 sec). This rapid increase gradually returned to basal levels within 60 sec. This stimulatory effect of hCG was dose-dependent, and the concentrations required were similar to those reported by us and others to stimulate steroidogenesis. Scatchard analysis revealed that in addition to the known high affinity (5.0 n*M*) benzodiazepine binding site, a second higher affinity (0.2 n*M*), hormone-induced, benzodiazepine binding site appeared. We then examined whether steroid synthesis could be detected in a similar time frame. MA-10 cells were incubated for 15 sec with aminoglutethimide, an inhibitor of P-450scc, together with hCG. Mitochondria were isolated from these cells and after incubation in aminoglutethimide-free buffer, an increase in the rate of pregnenolone formation was observed. Addition of a selective inhibitor of cAMP-dependent protein kinase blocked not only the hormone-induced PBR binding but also steroid formation. Furthermore, addition of flunitrazepam abolished the hCG-induced rapid stimulation of steroid synthesis. These results demonstrate that, in Leydig cells, the most rapid effect of hCG and cAMP, is the transient induction of a higher affinity benzodiazepine binding site, which occurs concomitantly with an increase in the rate of steroid formation (67). It should be noted that this biochemical evidence for the hormonal regulation of PBR is in agreement with the data presented above on the hormone-induced changes in PBR topography seen over the same time frame (43). This, in turn, suggests that hormones alter PBR to activate cholesterol delivery to the inner mitochondrial membrane and subsequent steroid formation.

In search of the mechanism underlying the effect of hCG and cAMP on PBR ligand binding, and considering the well-documented role of protein phosphorylation in the regulation of steroid biosynthesis, we identified putative phosphorylation motifs at the C-terminal domain of the cloned rat, bovine, and murine PBR protein. In mitochondrial preparations the cAMP-dependent protein kinase (but not other purified protein kinases) was found to phosphorylate the 18-kDa PBR protein (68). In addition, the 18-kDa PBR protein was found to be phosphorylated in digitonin-permeabilized Leydig cells, and its phosphorylation was stimulated by cAMP (68) suggesting that PBR are an *in vitro* and *in situ* substrate of the cAMP-dependent protein kinase. However, cloning of the human 18-kDa PBR protein predicted an amino acid sequence missing the phosphorylation motif identified in the rat, mouse, and bovine sequences, thus suggesting that phosphorylation of the 18-kDa PBR protein may not be an ubiquitous mechanism of regulation of PBR function. Thus, we have now turned our efforts to identifying PBR-associated proteins substrates of different kinases involved in the regulation of steroidogenesis.

A Constitutive Steroidogenic Cell Model for PBR Function in Steroidogenesis

In Leydig cell–derived tumors, steroid synthesis occurs independently of hormonal control since pituitary LH secretion is suppressed by the excessive amount of steroids produced. R2C cells are derived from rat Leydig tumors, and they maintain their *in vitro* capacity to synthesize ste-

roids constitutively in a hormone-independent manner (69). Thus, one can expect that constitutive steroidogenesis is driven by the unregulated expression of the hormonal mechanism that controls steroid synthesis or by an unknown separate mechanism. Radioligand binding assays on intact R2C cells revealed the presence of a single class of PBR binding sites with an affinity 10-times higher (Kd = 0.5 n*M*) than that displayed by the MA-10 PBR (Kd = 5 n*M*) (70). Photolabeling of R2C and MA-10 cell mitochondria with a photoactivatable PBR ligand showed that the 18-kDa PBR protein was specifically labeled. This indicates that the R2C cells express a PBR protein with properties similar to the MA-10 PBR. Moreover, a PBR synthetic ligand was able to increase steroid production in isolated mitochondria from R2C cells that express the 5 n*M* affinity receptor. Interestingly, mitochondrial PBR binding was increased 6-fold upon addition of the post-mitochondrial fraction, suggesting that a cytosolic factor modulates the binding properties of PBR in R2C cells and is responsible for the 0.5 n*M* affinity receptor seen in intact cells (70). The presence of a cytosolic steroidogenesis-stimulating factor in R2C cells has been reported (69), and the constitutive expression of the steroidogenic acute regulatory protein (StAR) by these cells has also been shown (71). In conclusion, these data demonstrate that ligand binding to the mitochondrial higher affinity PBR is involved in maintaining R2C constitutive steroidogenesis, and that a cytosolic protein may be involved in the regulation of this process.

A Bacterial Model for PBR Function in Cholesterol Transport

Bacteria present a model system without endogenous cholesterol. In addition, bacteria do not express PBR protein and ligand binding. *Escherichia coli* were transfected with mouse PBR cDNA in a PET vector. Addition of isopropyl-1-thiol-β-D-galactopyranoside (IPTG) to transfected bacteria resulted in the expression of the 18-kDa PBR protein and ligand binding with similar pharmacological characteristics to that previously described for PBR (72; Li and Papadopoulos, unpublished data). IPTG-induced PBR expression resulted in a protein-, and time-, and temperature-dependent uptake of radiolabeled cholesterol (Li and Papadopoulos, unpublished data). No uptake of other radiolabeled steroid could be seen. When IPTG-induced, cholesterol-loaded, bacterial membranes were treated with PK 11195, cholesterol was liberated from the membranes, suggesting that cholesterol is captured by PBR, which upon ligand binding releases cholesterol. Thus, PBR serves a channel function where cholesterol can freely enter and reside stored within the membrane, without being incorporated in the lipid bilayer. PBR ligand binding controls the opening/release state of the channel, thus mediating cholesterol movement across membranes.

The significance of this finding goes beyond the role of PBR in steroidogenesis. In addition to being a precursor for steroid hormone synthesis, cholesterol is an essential structural element of cellular membranes and a precursor for the synthesis of bile acids and lipoproteins. Mammalian cells obtain cholesterol by internalization of low density lipoproteins or by *de novo* synthesis in the endoplasmic reticulum. The subcellular distribution of cholesterol suggests that cholesterol is trafficked and incorporated quickly from the sites of acquisition to target membranes (73). Thus, a tissue- and cell-specific cholesterol homeostasis is achieved. Considering the widespread occurrence of PBR and their tissue- and cell-specific subcellular localization (7, 12, 13), these results suggest a more general role for PBR as a cholesterol channel in intracellular cholesterol transport and compartmentalization.

Targeted Disruption of the PBR Gene in Steroidogenic Cells

In order to investigate further the role of PBR in steroidogenesis, we developed a molecular approach based on the disruption of PBR gene in the constitutive steroid producing R2C rat Leydig cell line by homologous recombination (74; Papadopoulos, Amri, Li, Boujrad, Vidic, and Garnier, unpublished data). On the basis of the known rat PBR gene sequence, we designed two sets of primers that allowed us to amplify two fragments of the PBR gene from R2C cells genomic DNA by PCR. These PBR genomic DNA fragments were cloned and used to design the targeting construct. The targeting vector was constructed by positioning (i) the *neo* gene conferring the neomycin resistance, which allows for a positive selection of cells that undergo homologous recombination, in between the two PBR genomic DNA fragments and (ii) the Herpes Simplex Virus-tyrosine kinase gene, for the negative selection against cells that have randomly integrated the targeting construct, at the 3′-end of the second PBR genomic DNA fragment. The targeting vector was then transfected in R2C cells, and selection was performed with G418 and ganciclovir (75). A G418/Ganc-resistant cell line was generated. PBR expression, examined by ligand binding and immunoblot analysis, was absent in this cell line. In addition, the PBR-negative R2C cells produced minimal amounts (10%) of steroids compared to normal R2C cells. However, addition of the hydrosoluble analogue of cholesterol 22R-hydroxycholesterol, or transfection of the cells with a PBR cDNA, resulted in increased steroid production by the PBR-negative R2C cells, indicating that the cholesterol transport mechanism was impaired due to the absence of the 18-kDa PBR protein.

The 30-kDa steroidogenic acute regulator protein (StAR) is expressed in response to trophic hormones and in parallel with steroid production, and it has been suggested to be the labile mediator of the hormone-stimulated steroidogenesis (76). In addition, it has been postulated that constitutive steroidogenesis in R2C Leydig cells is maintained by the constitutive expression of StAR in these cells (71). We examined the presence of StAR in these cells using an antipeptide antiserum that we developed based on the pub-

lished amino acid sequence of the protein (77). Surprisingly, StAR levels remained the same in control and PBR negative cells where steroidogenesis ceased, suggesting that StAR may act before or at PBR to regulate steroid production.

In vitro Regulation of PBR Expression

To our knowledge, there have not been any reports on the regulation of the 18-kDa PBR protein expression. One of the reasons is that PBR are thought to function as a housekeeping gene, important for basic cell functions. Recently, we were able to alter PBR expression in Leydig cells using environmental toxicants (78; Boujrad and Papadopoulos, unpublished data). It has been shown that the compound perfluorodecanoic acid, a peroxisome proliferator and environmental hazard (used commercially as a surfactant in hydraulic fluids, heat exchangers, and film-coating foams) has antiandrogenic activity when administered to rats *in vivo* (79). We observed that, in MA-10 mouse tumor and rat Leydig cells, this compound inhibits hormone-stimulated biosynthesis in a dose- and time-dependent manner. The effect of perfluorodecanoic acid on steroidogenesis could be reversed, and the site of action of this compound was identified at the step of cholesterol transport to mitochondria, where we demonstrated that it disrupts steroidogenesis by reducing PBR ligand binding and protein expression. Interestingly, the expression of the StAR protein was not affected by the treatment with this toxic agent. Thus, PBR may be a tissue-specific target of environmental hazards, thus mediating their antisteroidogenic and antiandrogenic effect.

In vivo Models for PBR Function in Steroidogenesis

Efforts to generate a PBR-negative gene knockout mouse model failed, as the animals died at an early embryonic stage, suggesting that PBR are involved in basic functions necessary for embryonic development. Thus we turned our efforts to identifying a pharmacologic means of modulating PBR expression *in vivo*. Glucocorticoid excess has broad pathogenic potential including neurotoxicity, neuroendangerment, and immunosuppression. Glucocorticoid synthesis is regulated by ACTH, which acts by accelerating the transport of the precursor cholesterol to the mitochondria where steroidogenesis begins. *Ginkgo biloba* is one of the most ancient trees, and extracts from its leaves have been used in traditional medicine (80). A standardized extract of *Ginkgo biloba* leaves, termed EGb 761, has been shown to have neuroprotective and "antistress" effects. *In vivo* treatment of rats with EGb, and its bioactive component ginkgolide B (GKB), specifically reduces the ligand binding capacity, protein, and mRNA expression of the adrenocortical mitochondrial PBR (77). As expected, the ginkgolide-induced decrease in glucocorticoid levels resulted in increased ACTH release, which in turn induced the expression of the StAR protein. Since GKB reduced the adrenal PBR expression and corticosterone synthesis, despite the presence of high levels of StAR, these data demonstrate that PBR is indispensable for normal adrenal function. Moreover, these results suggest that manipulation of PBR expression could control circulating glucocorticoid levels and that the "antistress" and neuroprotective effects of EGb 761 are due to its effect on glucocorticoid biosynthesis.

We continued these studies with the goal to determine specific *ex vivo* effects of EGb 761 and GKB on isolated adrenocortical cells. Cells were isolated from rats treated with EGb 761, GKB, or saline. The effect of ACTH on normal and metabolically labeled cells was examined. Corticosterone levels were measured by RIA, and protein synthesis was analyzed by two-dimensional gel electrophoresis (2D-PAGE). Treatment with EGb 761 and GKB resulted, respectively, in a 60% and 80% reduction of corticosterone produced by the cells in response to ACTH, compared to cells isolated from saline-treated rats (81; Amri, Drieu and Papadopoulos, unpublished data). 2D-PAGE revealed that in cells from control and drug-treated animals ACTH induced at the same level the synthesis of the steroidogenic acute regulatory protein (StAR). In addition, treatment with EGb 761 and GKB specifically altered the synthesis of six proteins, including the inhibition of synthesis of a 17-kDa protein, identified as PBR. These results suggest that EGb 761 and GKB exert specific effects on adrenocortical cells because they affect specific protein synthesis, inhibit PBR expression, and reduce the ACTH-stimulated corticosterone production, despite the presence of the hormone-induced StAR. In conclusion, these studies indicate that EGb 761 and isolated ginkgolides may serve as the prototypes of a new generation of compounds regulating PBR expression. In addition, these results further demonstrate the determining role of PBR in cholesterol transport and steroidogenesis and further suggest that StAR may act at a step before the mitochondrial PBR.

Endogenous Ligands of PBR

In addition to the well-characterized drug ligands of PBR, two other entities were identified as endogenous PBR ligands: porphyrins (82) and the polypeptide diazepam binding inhibitor (DBI) (7, 83). Since in our model system porphyrins were found to have very low affinity for PBR and no effect on mitochondrial steroid formation (Papadopoulos, unpublished data) we focused our studies on the role of DBI. DBI is a 10-kDa protein originally purified from brain by monitoring its ability to displace diazepam from the allosteric modulatory sites for GABA action on $GABA_A$ receptors (84, 85). DBI was also independently purified and characterized for its ability to bind long-chain acyl-CoA-esters (86) and modulate insulin secretion (87). DBI was found to be present in a variety of tissues and highly expressed in steroidogenic cells (7).

Binding of DBI to PBR was initially determined by examining the ability of DBI to displace high-affinity radiolabeled PBR drug ligands (35, 88, 89). Competition stud-

ies for specific binding indicated that DBI displaced radiolabeled benzodiazepines. In addition, the stimulatory effect of DBI on steroid synthesis was specifically blocked by flunitrazepam, the PBR ligand shown to inhibit hormone-stimulated steroidogenesis (90). To further demonstrate that DBI binds specifically to PBR, we performed cross-linking studies on Leydig cell mitochondria using metabolically labeled bioactive [^{35}S]DBI (70). Two protein complexes were specifically labeled within R2C Leydig cell mitochondria. A protein complex with an apparent molecular size of 27 kDa, recognized by an antiserum against PBR, suggesting that the 10-kDa DBI formed a specific complex with the 18-kD PBR protein.

In search of a cytoplasmic steroidogenesis-stimulating factor(s), a protein of 8.2-kDa molecular size was isolated from bovine adrenals shown to stimulate transport of cholesterol into mitochondria and transport from the outer to the inner membrane (91). This 8.2-kDa protein was shown to be identical to DBI, except the loss of two amino acids (Gly-Ile) from the carboxy terminus (92), and to have a long half-life (93). We examined the effect of isolated 10-kDa DBI on mitochondria from adrenocortical and Leydig cells (90). Dose-response curves indicated that a 3-fold stimulation is obtained with low concentrations (0.1–1 μM) of DBI whereas higher concentrations have lower stimulatory effect on pregnenolone formation. The stimulation obtained was similar to those reported for the 8.2-kDa des-(Gly-Ile)-DBI on bovine adrenocortical mitochondria (91, 94). Moreover, similar results were obtained using purified rat and bovine testis DBI (35).

We then showed that the amino acid sequence 17 to 50 of the DBI bears the biological activity since the triacontatetraneuropeptide (TTN, DBI[17–50]) specifically stimulated mitochondrial steroidogenesis with a potency and efficacy similar to that of DBI (90). TTN together with other DBI peptide fragments were also found in adrenal and testis extracts, and we noted that DBI could be processed *in vitro* by mitochondria. Binding studies on mitochondria also indicated that TTN binds specifically to PBR (89). DBI and DBI processing products were also found to be present in brain and C6-2B glioma cell extract. DBI stimulated pregnenolone formation in mitochondrial fractions from C6-2B glioma cells and rat brain (55). In addition to DBI, the DBI peptide fragments DBI[17–50] and DBI[39–75] were found to be biologically active in *in vitro* assays (55, 57, 95).

Taking into account the findings that (i) hCG increases PBR ligand binding (38), (ii) DBI stimulates mitochondrial steroid formation acting *via* PBR (70, 90), and (iii) DBI is preferentially localized in the periphery of mitochondria (96), the possibility arises that trophic hormones, by altering PBR, increase PBR interaction with DBI; PBR-DBI interaction triggers steroidogenesis.

In order to determine the *in situ* role of DBI in steroidogenesis, we suppressed cell DBI levels using antisense oligodeoxynucleotides. In order to overcome the usually encountered oligodeoxynucleotide uptake problems, we took advantage of the ability of steroidogenic cells to utilize exogenous cholesterol *via* the lipoprotein endocytotic pathway (97). Thus, we constructed cholesterol-linked phosphorothioate oligodeoxynucleotides (CHOL-ODNs) complementary to either the sense or the antisense strand of the 24 nucleotides encoding mouse DBI, 9 bases immediately 5′ and 12 downstream to the initiation ATG codon. Treating MA-10 cells with CHOL-ODN antisense to DBI resulted in a dose-dependent reduction of DBI levels. In contrast, CHOL-ODN sense to DBI did not affect its expression. Saturating amounts of hCG increased MA-10 progesterone production by 150-fold. Addition of increasing concentrations of CHOL-ODNs sense to DBI or of a nonrelated sequence did not reduce the MA-10 response to hCG. In contrast, a 2-fold increase in the amount of steroids produced was observed due to the cholesterol linked to the ODN, liberated in the cells and used as substrate for steroid synthesis. However, in the presence of CHOL-ODN antisense to DBI, in amounts shown to reduce DBI levels, MA-10 cells lost their ability to respond to hCG. In these studies the hCG-stimulated cAMP levels and P-450scc activity were not affected by the CHOL-ODNs used (97).

Using similar technology we also decreased DBI levels in the R2C Leydig cells (70). DBI-depleted R2C cells did not produce steroids suggesting that DBI plays a vital role both in the acute stimulation of steroidogenesis by trophic hormones and in the constitutive steroid synthesis. Since we showed that DBI is not the long-sought labile factor, and that the site of hormone action is in the mitochondrion, we propose that hormones, by altering PBR, increase its interaction with DBI, which in turn, triggers steroidogenesis.

Although PBR drug ligands did not have any direct effect on P-450scc activity, examined in mitoplasts, DBI induced a 2-fold stimulation of pregnenolone synthesis. Evidence has been discussed already that indicates that the outer mitochondrial membrane PBR mediates the effects of PBR ligands and DBI on intact mitochondria. However, the observation that DBI stimulates pregnenolone production by inner mitochondrial membranes implies that this protein can also act *via* an additional PBR-independent mechanism. Further evidence indicating that the DBI acts directly on P-450scc was then provided by observations in an *in vitro* reconstituted enzyme system (98, 99) where DBI stimulated the production of pregnenolone suggesting that the non-PBR mechanism involved in steroidogenesis may involve direct activation of P-450scc or alternatively an indirect mechanism, which may act *via* increasing the availability of cholesterol or by altering the rate of reduction of P-450scc. It is evident that more work is needed in order to characterize the DBI–P-450scc interaction. In preliminary studies, we examined the distribution of DBI within the mitochondria and using anti-DBI antibodies, we identified the 10-kDa DBI in the outer mitochondrial membranes of adrenocortical cells but not in the inner membranes (83). However, in inner mitochondrial membrane preparations, we did observe a 30-kDa DBI-immunoreactive protein of 6.8 isoelec-

tric point. When adrenocortical cells were treated with ACTH for 30 min prior to the preparation of mitochondria, the levels of this 30-kDa protein within the inner mitochondrial membrane were reduced (83). The identity of this protein still remains unknown, as well as its relationship to the mature StAR protein. Due to the immunological homology of this protein with DBI, it is tempting to speculate that the 30-kDa DBI-related protein may be a higher molecular weight form of DBI that is processed by a hormone-dependent mechanism to release DBI directly at the inner mitochondrial membrane.

A New Tool to Study PBR Distribution and Function

Considering the data presented above, PBR is an attractive target molecule for the development of compounds that may be used for the regulation of steroid synthesis in the periphery and the CNS. However, the studies described above on the localization, structure, and function of PBR in fixed cells or isolated subcellular fractions were performed using high-affinity ligands, antisera developed against the entire molecule or fragments of the 18-kDa PBR protein, and cDNA probes. Considering the problems associated with antiserum specificity, sensitivity, stability, and tissue penetration, as well as with subcellular fractionation, one could argue that the results obtained may not represent the actual situation in a living cell. We recently reported the synthesis, characterization, and biologic activity of a fluorescent high-affinity PBR ligand (compound 4). Based on the structure of previously described high-affinity drug ligands for the peripheral-type benzodiazepine receptor, a 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) derivative of 2-phenylindole-3-acetamide (compound 4) was synthesized (100). This fluorescent probe displaced the radiolabeled isoquinoline PK 11195 from the isoquinoline binding site on the 18-kDa PBR protein with a Ki of 10 n*M*. Compound 4 expressed a full steroidogenesis-activating property when added to mitochondria of both testis Leydig and brain glial cells, as described for high-affinity PBR ligands. In addition, compound 4 specifically labeled the intracellular localization of PBR in a manner consistent with the previously reported localization of PBR in these cell types. Thus, compound 4 provides a tool allowing the direct imaging by fluorescence microscopy of the 18-kDa PBR protein in living cells and a tool to probe the localization and function of PBR in different tissues and under conditions where antisera cannot penetrate.

Interaction of PBR with Other Proteins and StAR

Considering the interaction of 18-kDa PBR with VDAC at the contact-site level, we will have to consider a potential role of other proteins shown to participate in contact-site formation. The inner mitochondrial membrane adenine nucleotide carrier was previously shown to be structurally associated with PBR components (38). The inner mitochondrial megachannel (IMC) (101), may coincide with the permeability transition pore or the multiple conductance channel activity (102) (channels identified using the patch clamp technology) located in the inner membrane of the contact sites and represent activities regulated by voltage and ion (i.e., calcium) changes that result in pore opening and permeability increases. VDAC was shown to be part of the IMC (103). IMC is inhibited by PBR ligands (104) and is sensitive to the immunosuppressant cyclosporin A (101). Interestingly, we observed that cyclosporin A is a noncompetitive inhibitor of PBR, suggesting that IMC may regulate PBR ligand binding and function in an "allosteric" manner (Papadopoulos, unpublished data). Considering these observations, we propose a model of the PBR complex, present at the contact sites of mitochondrial membranes, composed of the 18-kDa PBR protein, VDAC, and two inner-membrane proteins, adenine nucleotide carrier and IMC.

As noted above, StAR has been found only in gonadal and adrenal cells where it is newly synthesized, in response to trophic hormones and cAMP, as a cytoplasmic precursor protein of 37 kDa targeted to mitochondria (76). StAR synthesis parallels the capacity of the cells to produce steroids in response to trophic hormones, and expression of StAR in the absence of hormonal stimulation resulted in a 3-fold increase in progesterone production by MA-10 Leydig cells (76). The 37-kDa StAR precursor further undergoes cleavage to produce the 30-kDa mitochondrial "mature" StAR protein and its phosphorylated counterpart. This protein processing is believed to occur at the level of the outer/inner mitochondrial membrane contact sites and has been proposed to be responsible for cholesterol transport from the outer to the inner mitochondrial membrane (76). However, recent studies demonstrated that StAR does not need to enter into mitochondria to stimulate steroidogenesis and that it may function by activating a mitochondrial receptor or transport mechanism (105). Thus, it is likely that StAR protein may be stimulating cholesterol delivery to P-450scc by acting on PBR either directly or *via* a PBR-associated protein. This hypothesis is further supported by the data presented above in the experiments using the PBR-negative Leydig cells and the *in vivo* studies.

We directly tested the hypothesis that StAR may interact with the 18-kDa PBR protein. This protein-protein interaction was examined using the yeast two-hybrid system (106). In this system, the Ga14 transcription factors can be functionally and physically divided into a DNA-binding domain and a transcription-activation domain. Linking these two domains to other proteins results in chimeric proteins with either specific DNA-binding ability or a nonspecific transcription-activation function. Interaction between these two chimeric proteins in yeast can bring the Ga14 activation domain and the DNA binding domain together and reconstitute the Ga14 transcription-activation function. Using this model, we constructed a PBR and StAR chimeric proteins (Li and Papadopoulos, unpublished data). No direct PBR-StAR interaction could be seen. However, this finding does

not exclude the possibility that a mediator protein may be required for this interaction.

Conclusion

PBR seems to serve the function of cholesterol channel in different tissues. In the specialized steroidogenic tissues (adrenals, gonads, placenta, and brain), this function is associated with increased steroid synthesis. The specificity of the role of PBR in steroidogenic cells comes from the observations that PBR is highly expressed in steroidogenic tissues where it is localized primarily in the outer mitochondrial membrane, its affinity and topography are regulated by peptide trophic hormones, and PBR ligands stimulate cholesterol transport and steroid synthesis. Although this is a hormone-regulated process in adrenals and gonads, in placenta and brain this process seems to be constitutive or developmentally regulated. Alternatively, a common third messenger system may exist in all steroidogenic tissues that respond to tissue-specific second messenger systems. A good candidate for this function is Cl^- anion movement. There is evidence for the presence of Cl^- channels in steroidogenic cells (107), and for the role of Cl^- ions in steroid (108, 109) and neurosteroid (110) biosynthesis. VDAC is an anionic channel, and ion channel blockers were shown to inhibit PBR ligand binding (111) and steroidogenesis (108). Thus, we can envision a regulatory pathway where tissue-specific second messenger systems, either directly or *via* a common third messenger, modulate the characteristics of the mitochondrial PBR complex and/or the interaction of PBR with other mitochondrial or cytosolic proteins, resulting in cholesterol movement across the mitochondrial membranes. In this scheme, PBR are the "gate keeper" controlling the rate of steroid synthesis. However, this hypothesis remains to be explored.

Considering the widespread occurrence of PBR and their tissue- and cell-specific subcellular localization, this specificity is achieved by a yet unknown mechanism. However, the data on the interaction of PBR with VDAC and on the role of the lipid milieu on PBR ligand binding (25, 112, 113) clearly demonstrate the role of the microenvironment on PBR function. Thus, we believe that tissue- and cell-specific PBR-associated proteins (PAPs) play a regulatory role on PBR function. We recently used PBR as the bait to screen a mouse testis cDNA library in order to identify PAPs. Using this method, we isolated the cDNAs for eight PAPs (Li and Papadopoulos, unpublished data). The characterization and interaction of these proteins with PBR is under investigation. Preliminary data indicate that two of these proteins may be the second messenger system–dependent mediator of the PBR activation responsible for the induction of the expression of high-affinity PBR, the changes seen in PBR topography, and the initiation of cholesterol movement into mitochondria. It is possible that a PAP may also be the mediator of a StAR-PBR interaction responsible for the continuous supply of the steroidogenic machinery with cholesterol which, through PBR, will reach the P-450scc.

Thus, elucidation of the structure of the PBR complex in steroidogenic cell mitochondria may be the key step leading to the identification of the sequence of events leading to cholesterol movement into the mitochondria and the subsequent steroid formation.

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