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Peripheral-Type Benzodiazepine Receptor

Role in the Regulation of Steroid and Neurosteroid Biosynthesis

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INTRODUCTION

Eukaryotic steroid hormones, derived from cholesterol, are involved in the maintenance of the organism's homeostasis, adaptability to the environment, and developmental and reproductive functions. In addition to the well-defined actions in peripheral tissues, steroids have pleiotropic actions on the central nervous system (CNS), where they control a number of neuroendocrine and behavioral functions. Thus, comprehension of the molecular systems underlying the control of steroid hormone biosynthesis is essential for the study and treatment of a multitude of physiological disorders.

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PERIPHERAL-TYPE BENZODIAZEPINE RECEPTORS (PBRs)

Pharmacological and Biochemical Characteristics

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 CNS *(1,2)*. However, in search of specific binding sites for benzodiazepines outside the CNS another class of binding sites was first observed in the kidney *(3)* and later determined to be present in apparently all tissues including the CNS *(4–7)*. This class of binding sites is commonly referred to as the peripheral-type benzodiazepine recognition sites or receptors (PBRs) owing to its initial discovery in peripheral tissues.

PBRs were distinguished from $GABA_A$/benzodiazepine receptors by several criteria. Although both receptors bind diazepam with relatively high affinity, they exhibited very different binding specificities. In rodent species, PBRs bind 4'-chlorodiazepam with high affinity, whereas $GABA_A$ receptors show low affinity for this benzodiazepine *(6,7)*. Conversely, clonazepam and flumazenil, which bind with high affinity to $GABA_A$ receptors, exhibit very low affinities for PBR. PBR also bind with high affinity the imidazopyridine alpidem and has a low affinity for the imidazopyridine zolpidem *(8)*. On the contrary, $GABA_A$ receptors bind with high-affinity zolpidem and have low affinity for alpidem. In addition, PBRs have high affinity for three classes of compounds, isoquinoline *(9)*, indoleacetamide *(10)*, and pyrrolobenzoxazepine *(11)* derivatives, which do not bind to the $GABA_A$ receptors *(10,11)*. Isoquinolines were the major tool used for the identification and characterization of PBR *(5–7)*. In addition to these differences in drug specificity, it has been well-established that $GABA_A$/benzodiazepine receptors, composed of 50–55 kDa protein subunits, are coupled to synaptosomal chloride channels whereas PBR, an 18 kDa protein associated with other mitochondrial proteins, are not coupled to GABA recognition sites and their function will be addressed in this review. Subcellular fractionation studies demonstrated that PBRs were primarily localized on mitochondria *(12–14)*, and more specifically on the outer mitochondrial membrane *(15)*, although it is likely that they are not exclusive to this organelle. A plasma membrane location for this receptor was recently identified *(16–18)*.

The first identification of a molecular component associated with PBRs was made possible by the development of a photoaffinity probe, the isoquinoline propanamide PK 14105 *(19)*. This probe specifically labeled an 18 kDa protein, which was subsequently purified *(20,21)*, and the corresponding cDNA cloned from rat *(22)*, human *(23,24)*, bovine *(25)*, and murine *(26)* species. The cDNA sequence of the 18 kDa protein specifies an open reading frame of 169 aminoacids 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 domains for PBR ligands although, owing to the constitutive expression of PBRs in all cells used for transfections, 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 PBRs, the affinity of benzodiazepines for PBRs is species-specific, varying from high affinity (rodents) to low affinity (bovine) *(6,7)*. These species differences in benzodiazepine binding may be also owing to either structural differences in the 18 kDa protein or to 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 *(27,28)*. The gene encoding the 18 kDa PBR protein has been isolated and characterized for rat *(29)* and human *(24)*. In both species, the gene contains four exons spanning 10–13 kb and the locations of the introns are identical.

One of the interesting features of the 18 kDa PBR protein is the observation that, although this protein is targeted to the mitochondria, it does not contain a typical mitochondria-targeting signal sequence. 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 *(26)*. 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 owing 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 *(26)*. 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 30–35 kDa protein, nonspecifically labeled using irreversible isoquinolines and benzodiazepines, to be associated with PBR *(4–7)*. Among the ligands used to identify this 30–35 kDa protein was flunitrazepam *(4–7)*. 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 which inhibit VDAC function were able to abolish PBR ligand binding, the hypothesis that VDAC was part of PBR was advanced *(4)*. Moreover, we observed that, among the PBR ligands tested, only flunitrazepam could specifically antagonize the hormone-stimulated cholesterol transport and steroidogenesis, acting via PBR *(30)*. 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 (ADC) *(31)* suggested that PBR is not a single protein receptor but a multimeric complex.

These studies demonstrated that VDAC is associated functionally to the 18 kDa PBR and is part of the benzodiazepine binding site in the PBR. Benzodiazepine binding, however, 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 owing to a five nonconserved amino acids in the C-terminal end of the 18 kDa PBR protein *(32)*. 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 3nm, 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 *(33)*. 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 *(33,34)*.

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 ADC was previously shown to be structurally associated with PBR components *(31)*. The inner mitochondrial megachannel (IMC) is also located in the inner membrane of the contact sites and represents activities regulated by voltage and ion (i.e., calcium) changes that result in pore opening and permeability increases *(35,36)*. IMC is inhibited by PBR ligands *(37)* and is sensitive to the immunosuppressant cyclosporin A *(35)*. Interestingly, we observed that cyclosporin A is a noncompetitive inhibitor of PBR, suggesting that IMC may regulate PBR in an "allosteric" manner (Papadopoulos, unpublished data). Taken together, 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, ADC and IMC.

PBR Topography in Mitochondrial Membranes

Native MA-10 Leydig tumor cell mitochondrial preparations were examined by transmission electron and atomic force microscopic (AFM) procedures in order to investigate the topography and organization of PBR. Mitochondria were immunolabeled with an anti-PBR antiserum coupled to gold-labeled secondary antibodies. Results obtained indicated that the 18 kDa PBR protein is organized in clusters of 4–6 molecules *(38)*. Moreover, in many occasions, the interrelationship among the PBR molecules was found to favor the formation of a single pore. Because the 18 kDa PBR protein is associated functionally 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. Fifteen-s treatment with hCG induced the appearance of large clusters of gold labeled PBR 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 *(39)*. 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 *(39)*. 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 *(40)*. 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 should be also noted that intramitochondrial translocation of phospholipids occurs in a similar manner through mitochondrial contact sites *(41)*.

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 *(42,43)*. 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 "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, presented in the section "PBR in Steroid Biosynthesis," on the role of PBR in the intramitochondrial cholesterol transport.

STEROID BIOSYNTHESIS

Trophic hormone regulation of steroid synthesis can be thought as being either "acute"—occurring within minutes and results in the rapid synthesis of steroids, or "chronic"—occurring over a long period of time and resulting in continued steroid production. The primary point of control in the acute stimulation of steroidogenesis by hormones involves the first step in this biosynthetic pathway, where the substrate cholesterol is converted to pregnenolone (PREG) by the cholesterol side-chain cleavage cytochrome P-450 enzyme (P-450scc) and auxiliary electron-transferring proteins, localized on inner mitochondrial membranes *(44–47)*. More 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 P450scc active site *(44–47)*. This hormone-dependent transport mechanism was shown to be mediated by cyclic adenosine monophosphate (cAMP), to be regulated by a cytoplasmic protein, and to be localized in the mitochondrion, where it regulates the intramitochondrial transport of cholesterol *(44–47)*. Although a number of molecules have been proposed as potential candidates mediating this intramitochondrial cholesterol transfer *(46,47)*, no clear evidence has been presented on the identity of this mechanism. During the last decade, however, a new cholesterol-transport mechanism was identified and characterized as mediating the acute stimulation of steroidogenesis by hormones, the PBR protein *(6)*.

PBR IN STEROID BIOSYNTHESIS

Effects of Drug Ligands

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 *(5,6)*. 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 PREG 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 *(45,47)*. 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 PREG 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 PREG formation was owing to PBR-mediated translocation of cholesterol from the outer to the inner mitochondrial membrane *(50)*. PBR ligands, however, induced a massive translocation of cholesterol: 10 μg/mg of protein. Considering that only approx 10–20% of this cholesterol will be used for steroidogenesis, these data indicate that PBR-mediated lipid translocation may be also involved in a more general mechanism, such as mitochondrial 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 have corroborated these observations *(51,52)* and extended them to placental *(53)* and ovarian granulosa cells *(54)*. Moreover, we showed that a similar mechanism regulates brain glial cell neurosteroid synthesis (*55*; *see* the section "PBR in Neurosteroid Biosynthesis"). Recently a PBR-mediated cholesterol transport mechanism was also identified in rat liver mitochondria *(56)*. Thus, it seems that the regulation of intramitochondrial cholesterol transport may be a general function of PBR.

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 *(30)*. 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 PREG 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. It should be noted, however, 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 h with hCG was without any effect on PBR binding or message levels *(57)*. However, addition of hCG to MA-10 cells resulted in a very rapid increase of PBR binding capacity (threefold increase within 15 s). This rapid increase gradually returned to basal levels within 60 s. This stimulatory effect of hCG was dose-dependent and the concentrations required were similar to those reported by us and others to stimulate steroidogenesis *(57)*. 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 s 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 PREG 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 *(57)*. It should be noted that this biochemical evidence for the hormonal regulation of PBR is in agreement with the data previously presented on the hormone-induced changes in PBR topography seen over the same time frame *(39)*. 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 *(58)*. In addition, the 18 kDa PBR protein was found to be phosphorylated in digitonin-permeabilized Leydig cells and its phosphorylation was stimulated by cAMP *(58)*, suggesting that PBR is an in vitro and *in situ* substrate of PKA. 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 in identifying PBR-associated proteins substrates of PKA.

PBR in a Constitutive Steroid-Producing Cell Model

In Leydig cell-derived tumors, steroid synthesis occurs independently of hormonal control, because pituitary LH secretion is suppressed by the excessive amount of steroids produced *(59)*. R2C cells are derived from rat Leydig tumors and maintain their in vitro capacity to synthesize steroids constitutively in a hormone-independent manner *(60)*. 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*) *(61)*. 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 that has 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 nM affinity receptor. Interestingly, mitochondrial PBR binding was increased sixfold 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 *(61)*. In conclusion, these data demonstrate that ligand binding to the mitochondrial higher affinity PBR is involved in maintaining R2C constitutive steroidogenesis.

PBR-Mediated Cholesterol Transport in Bacteria

Bacteria is a model system without endogenous cholesterol. In addition, bacteria do not express PBR protein and ligand binding. *Escherichia coli* were transformed 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 *(61a)*. IPTG-induced PBR expression resulted in a protein-, time-, and temperature-dependent uptake of radiolabeled cholesterol. 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 releases cholesterol upon ligand binding. 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.

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 *(61b)*. 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 that allows for a positive selection of cells that have undergone

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 *(62)*. Four G418/Ganc-resistant cell lines were generated. PBR expression, examined by ligand binding, was absent in all four cell lines. In addition, the PBR-negative R2C cells produced minimal amounts (10%) of steroids compared with normal R2C cells. However, incubation with the hydrosoluble analog of cholesterol, 22R-hydroxycholesterol, increased the steroid production by the PBR-negative R2C cells, indicating that the cholesterol-transport mechanism was impaired. The genomic DNA characterization of the PBR-negative R2C cells is under investigation.

Role of PBR in In Vivo Steroidogenesis

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 known, and extracts from its leaves have been used in traditional medicine *(63)*. A standardized extract of Ginkgo biloba leaves, termed EGb 761 (EGb), has been shown to have neuroprotective and "antistress" effects. In vivo treatment of rats with EGb, and its bioactive components ginkgolides A and B, specifically reduces the ligand-binding capacity, protein, and mRNA expression of the adrenocortical mitochondrial PBR *(64)*. As expected, the ginkgolide-induced decrease in glucocorticoid levels resulted in increased ACTH release which in turn induced the expression of the steroidogenic acute regulatory protein (StAR). Because ginkgolides 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 are owing to its effect on glucocorticoid biosynthesis. In addition, these data indicate that EGb and isolated ginkgolides may serve as the prototypes of a new generation of compounds regulating PBR expression.

NEUROSTEROID BIOSYNTHESIS

The specific interactions of steroids with binding sites at neuronal membranes and the ability of various steroids to modulate the brain function has prompted the investigation of the steroidogenic potential of CNS structures. The pioneering work of Baulieu et al. *(65)* demonstrated that glial cells can convert cholesterol to PREG and give origin to steroid metabolites, which are potential modulators of neuronal function. It has been shown that oligodendrocytes, a glioma cell line, and Schwann cells have the ability to metabolize cholesterol to PREG, the first step in steroid biosynthesis. However, discrepancies exist among the levels of the enzymatic activity, the amount of immunoreactive protein, and mRNA of the P450scc present in brain. In addition, despite the high levels of DHEA (the first neurosteroid described) found in brain, no one has demonstrated the presence of the 17α-hydroxylase cytochrome P450 activity in brain (P450c17). Thus, it

seems that brain steroid synthesis may not fit the well-defined scheme of adrenal, gonadal, and placental steroidogenesis, and that new pathways should be explored. Understanding the mechanisms of PREG and DHEA formation is paramount to all speculation and hypotheses about neurosteroids and their role in brain function. The levels of these steroids and their sulfated and lipoidal forms in brain are distinct from the peripheral steroid levels *(66,67)*, and their function as neuroactive steroids at the $GABA_A$ and NMDA receptor level has been well-established *(68)*.

We examined neurosteroid synthesis using the C6-2B subclone of the rat C6 glioma cell line. Figure 1 shows that these cells express both the glial fibrillary acidic protein (GFAP), a general marker for glial cells in culture, and galactocerebroside C (Gal C), a specific cell-surface marker for oligodendrocytes in culture. In agreement with our previous findings, using isolated mitochondria in immunoblot studies *(55)*, C6-2B cells also express P450scc protein, but not the P450c17 protein. We then demonstrated that these cells were able to synthesize PREG from the substrates mevanolactone and cholesterol *(69)*.

In order to examine the activity of the P-450scc present in C6 cells hydroxylated analogs of cholesterol were used, which will freely cross the mitochondrial membranes thus accessing the P-450scc in the inner membrane. Three different hydroxylated cholesterols (25-, 22-, and 20-OH-cholesterol) stimulated mitochondrial production of PREG by three to fivefold. Glial-cell P-450scc activity was also tested with hydroxylated cholesterol analogs in the presence of aminoglutethimide, an inhibitor of adrenal, testis, and ovarian P-450scc. Aminoglutethimide inhibited the PREG formation in a concentration-dependent manner from 25- and 22-OH-cholesterol, but failed to affect the conversion of 20-OH-cholesterol into PREG *(55)*. These findings suggest a functional analogy between adrenal and glial P-450scc. Thus, in addition to the P450scc protein, C6-2B cells also express P450scc activity. More recently, we demonstrated that human glioma cells in culture are also able to synthesize neurosteroids in a similar manner to C6-2B cells, thus validating the use of the C6-2B cell model system. Although C6-2B glial cell P450scc protein levels closely correlated those found in the adrenal gland, the P450scc mRNA was undetectable by Northern-blot and RNase protection assays. P450scc mRNA could be detected only after 35 cycles of PCR. Furthermore, the P450scc enzymatic activity in glial cells was 10-fold less than the activity of the adrenal enzyme. These discrepancies may be owing to differences in transcriptional regulation, mRNA stability, and antibody specificity and affinity. Alternatively, the possibility exists that neurosteroids, all or in part, may be formed from alternative precursors using alternative pathways *(70,71)*.

It is important to note that P450scc activity is related to the oligodendrocyte differentiation process *(72)*. Cholesterol accumulation in brain is also related to differentiation *(73)* and coincides with the rate of myelinization *(74)*. Interestingly, all three activities reach their maximum in the rat at 20 d of age and cholesterol accumulation in brain declines after maturation of the CNS structures, such as myelin and nerve endings. These findings demonstrate a temporal relationship among cholesterol accumulation, steroid synthesis, myelinization, and nerve-ending formation. The functional consequence(s) of this relationship is under investigation.

Despite the overwhelming evidence that P450scc activity is localized to glial cells of the brain, an earlier study demonstrated the presence of P450scc immunoreactivity in select neuronal populations *(75)* and more recently P450scc mRNA was detected by the

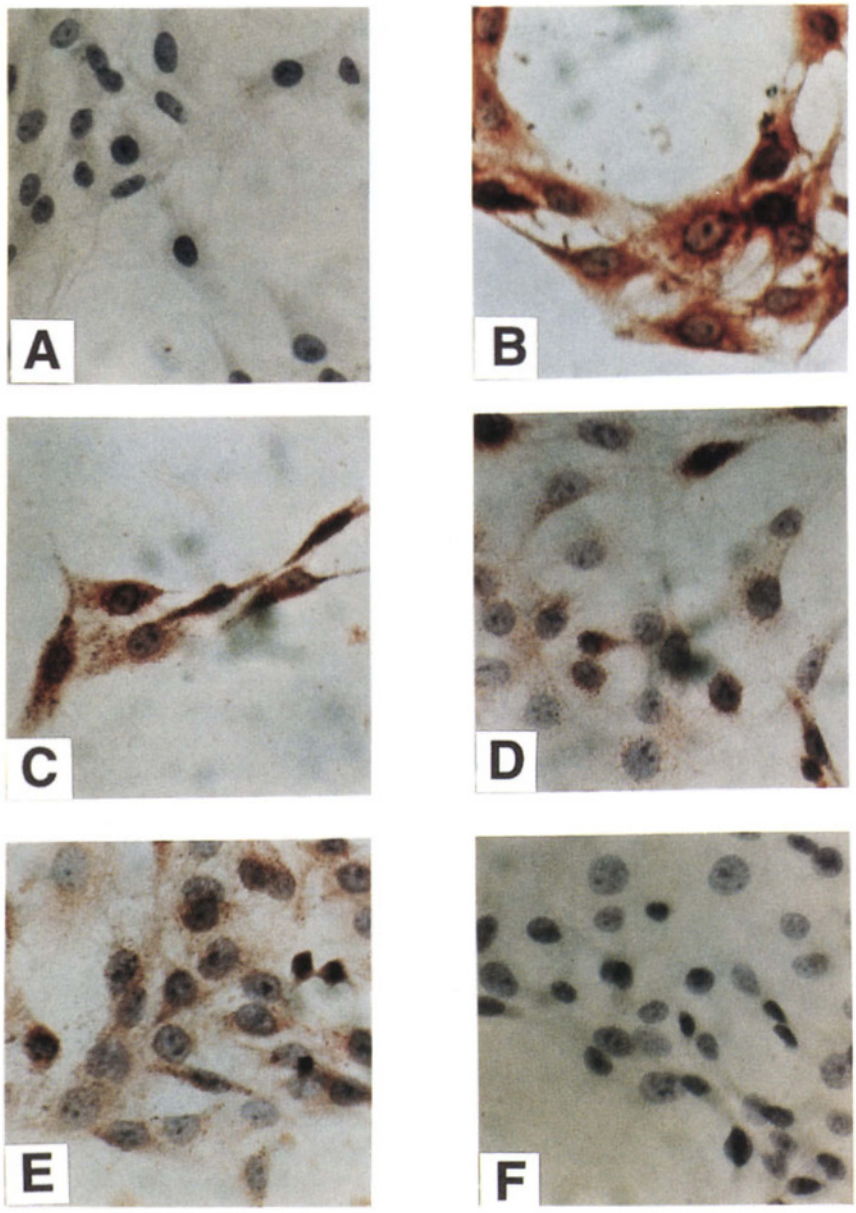

Fig. 1. Characterization of C6-2B rat tumor glioma cells. Cells were immunostained with antibodies: **(B)** anti-GFAP(1:160); **(C)** anti-GALC (1:100); **(D)** anti-PBR (1:200); **(E)** anti-P450scc (1:200); and **(F)** anti-P450c17 (1:200). Normal rabbit serum control is shown in **(A)**. Horseradish-peroxidase conjugated secondary antibody was used for the detection of the anti-serum-antigen complex.

polymerase chain reaction (PCR) technique in primary cerebellar granule neurons *(76)*. In addition, using isolated adult rat retina as a model system, we observed that the neuronal ganglion cells express P450scc protein and activity, and thus are able to synthesize steroids *(77)*.

Regulation of Neurosteroid Biosynthesis by cAMP

In order to understand the mechanisms of neurosteroid synthesis, we looked at well-established mechanisms in peripheral steroidogenic tissues. As previously noted, the regulation of steroid synthesis can be thought of as being either "acute," (occurring within minutes), or "chronic," (occurring over a long period of time). In the chronic regulation, peptide hormones and cAMP act by inducing the expression of steroidogenic enzymes. In acute regulation, the rate of steroid formation depends on the rate of cholesterol transport from intracellular stores to the inner mitochondrial membrane and loading of the P450scc with cholesterol. Using the C6-2B glioma cell line, we demonstrated the presence of both regulatory mechanisms—cAMP and mitochondrial PBR—in the control of neurosteroid synthesis.

Mitochondria isolated from C6-2B cells incubated with the P450scc inhibitor aminoglutethimide and the cAMP analog $(Bt)_2$cAMP showed greater rates of side-chain cleavage than mitochondria from cells incubated with aminoglutethimide alone *(78)*. After 6 h incubation with $(Bt)_2$cAMP, a significant increase was observed that increased over time to twofold at 24 h. A dose-response curve for $(Bt)_2$cAMP showed a significant increase using concentrations higher that 0.1 m*M*. Another cAMP analog (8-Br-cAMP) and agents known to increase cAMP levels, such as cholera toxin (an activator of the Gsα protein), forskolin, an activator of the catalytic subunit of adenylyl cyclase, and isoproterenol (a β-adrenergic receptor agonist), were found to stimulate the rate of side-chain cleavage in mitochondria *(78)*. All these data demonstrate that cAMP stimulates C6 glial cell PREG formation. Because all the stimuli used increase cAMP synthesis or mimic cAMP, the amount of PREG formed in their presence represents the maximal rate of cholesterol transport and metabolism under the conditions used. Using the Rp diastereoisomer of adenosine 3',5'-cyclic phosphorothioate (Rp-cAMPS), an antagonist of the activation of cAMP-dependent protein kinase, an inhibition of the isoproterenol-stimulated rate of mitochondrial side-chain cleavage was observed, indicating that isoproterenol is acting via cAMP and cAMP-dependent protein kinase to stimulate C6-2B glial cell PREG formation. These results demonstrated that neurosteroid synthesis is regulated by β-adrenergic receptor agonists and intracellular second messenger systems (cAMP). Considering the time of the response (>12 h) these data suggest that the effects seen may be owing to a direct effect of cAMP on the expression of steroidogenic enzymes or other components of the steroidogenic machinery, including cholesterol de-esterification and use for steroidogenesis. These findings were recently extended by Zhang and colleagues *(79)*, who demonstrated that cAMP stimulates P450scc transcription in C6 glioma cells.

Considering that the amount of steroids synthesized depends on the amount of the substrate cholesterol available, we performed a series of experiments in the presence of the early precursor of cholesterol, mevalonolactone. As expected, following 10 min of incubation with mevalonolactone, steroidogenesis increased, indicating that glia cells have limited sources of endogenous cholesterol required to support a continuous steroid production. Addition of isoproterenol further increased (doubled) the amount of steroids synthesized, suggesting that an acute stimulation of the adenylyl cyclase by isoproterenol increased glial cell steroid formation by controlling an early step of the pathway *(78)*. This control may be localized either at the level of the StAR protein, a hormone-dependent protein found in steroid synthesizing tissues *(80)*, or at the level of the mitochondrial PBR *(6)*. Because StAR has not been found in brain *(80)*, and hormones and cAMP were

found to induce rapid biochemical and morphological changes in the mitochondrial PBR *(39,57)*, this receptor is, at present, the only candidate to mediate the neurosteroidogenic effect of cAMP.

PBR in Neurosteroid Biosynthesis

We have 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 *(14,81)*. Ligand-binding studies indicated that rat brain mitochondria contain approximately 1 pmol/mg protein PK 11195 binding sites. In contrast, mitochondria from primary glial cells and C6-2B glioma cells exhibited a very high density for PBR (25–50 pmol/mg protein). This single class of binding sites had an apparent Kd of 4 n*M*. Photolabeling with the radiolabeled isoquinoline PK 14105 confirmed that brain, primary glial, and C6-2B glioma cell PBR is characterized by the 18 kDa protein, similar to that identified in peripheral tissues *(14,81)*, recognized by an anti-PBR peptide antisera that we developed (*64*; Fig. 1). In addition, the glioma receptor expressed identical pharmacological profile to the adrenocortical and testicular Leydig-cell PBR. These findings demonstrated that within the CNS, PBR is found primarily in glial cells. In addition, the mitochondrial localization and density of PBR in glial cells suggest that it may serve a function similar to that seen in peripheral steroidogenic tissues. Interestingly, the PBR immunolocalization in C6-2B glioma cells was similar to that seen for P450scc (Fig. 1).

We then investigated whether PBR ligands affect PREG formation in C6-2B glial cell mitochondria. At nanomolar concentrations, PK 11195 and Ro5-4864 induced a twofold stimulation of mitochondrial steroid production *(55)*. A similar increase was obtained with anxiolytic benzodiazepines that bind to both class 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. Table 1 shows a summary of the data obtained using mitochondria from C6-2B glioma cells *(55)* compared to Leydig and adrenal cell mitochondria *(49,50)* and to rat brain mitochondria from two separate studies *(82,83)*. It is obvious that, in our hands and using the same methods, glial cell mitochondria have a rate of PREG production 10 times slower than adrenocortical or Leydig cell mitochondria. However, in all three models, PBR drug ligands stimulated the rate of pregnenolone formation by two- to threefold. In two separate studies, rat brain mitochondria had a greater rate of PREG formation *(82,83)*, although in another report the rate of PREG formation by rat brain mitochondria was found identical to that seen in C6-2B glial mitochondria (Table 2, *84*). In these studies, PBR drug ligands stimulated PREG formation by three to fivefold.

Biosynthesis of [^{3}H]cholesterol and [^{3}H]PREG was demonstrated in C6-2B glial cell cultures to occur within seconds upon addition of the precursor [^{3}H]mevalonolactone *(69)*. Addition of 100 nM Ro5-4864 resulted in 141 and 205% increases in cholesterol and PREG formation, respectively *(69)*. This effect of Ro5-4864 was dose- and time-dependent and demonstrated that PBR ligands also stimulate steroid formation in cultured glial cells.

Table 1
Rate of Pregnenolone Formation in Response to PBR Drug Ligands

Mitochondria	*Pregnenolone, pmol/mg protein/min*					
	Treatment (1 μM)					
	Ref.	*Vehicle*	*Clonazepam*	*PK 11195*	*Ro5-4864*	*FGIN*
MA-10 Leydig cell	*(49)*	15	13	40	38	42
Y-1 Adrenal cell	*(50)*	8.1	8.0	21	20	18
C6-2B glioma cells	*(55)*	1.0	1.1	2.3	2.0	2.2
Rat brain	*(82)*	21	29	175	84	ND
Rat brain	*(83)*	27	27	ND	145	150

Ro5-4864, 4'-chlorodiazepam; PK 11195, 1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide; FGIN (FGIN-1-27), 2-hexyl-indole-3-acetamide. ND, not determined.

Table 2
Rate of Pregnenolone Formation in Response to Endogenous PBR Ligands

Mitochondria	*Pregnenolone, pmol/mg protein/min*			
	Treatment (1 μM)			
	Ref.	*Vehicle*	*DBI*	*Protoporhyrin IX*
MA-10 Leydig cell	*(94)*	15	40	16
Y-1 Adrenal cell	*(94)*	8.1	18	8.4
C6-2B glioma cells	*(55)*	1.0	2.2	1.3
Rat brain	*(82)*	21	40	ND
Rat brain	*(84)*	1.0	2.2	ND

ND, not determined.

In addition to the in vitro and in situ studies previously presented, PBR drug ligands were found to increase rat forebrain pregnenolone synthesis in vivo *(85)* 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.

ENDOGENOUS LIGANDS OF PBR

In addition to the well-characterized drug ligands of PBR two other entities were identified as endogenous PBR ligands, porphyrins *(86)* and the polypeptide diazepam binding inhibitor (DBI) *(6,87)*. Because in our model system porphyrins were found to have very low affinity for PBR and no effect on mitochondrial steroid formation (Table 2), 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 *(88,89)*. DBI was also independently purified and characterized for its ability to bind long-chain acyl-CoA-esters *(90)* and modulate insulin secretion *(91)*. DBI was found to be present in a variety of tissues and to be highly expressed in steroidogenic cells *(6)*.

DBI Acts via PBR

Binding of DBI to PBR was initially determined by examining the ability of DBI to displace high-affinity radiolabeled PBR drug ligands *(18,92,93)*. Competition studies for specific binding indicated that DBI displaced radiolabeled benzodiazepines with an inhibitory constant of 1–2 μ*M*. In subsequent studies, we analyzed the binding of DBI to PBR under conditions identical to those used to examine DBI effects on mitochondrial steroid synthesis *(55)*. We found that the inhibitory constant of DBI for PBR binding inhibition was 100 n*M*, a value that correlates well with the EC_{50} of DBI for mitochondrial steroid synthesis induction. In addition, the stimulatory effect of DBI on steroid synthesis (*see* the following section) was specifically blocked by flunitrazepam, the PBR ligand shown to inhibit hormone-stimulated steroidogenesis *(94)*. To further demonstrate that DBI specifically binds to PBR, we performed crosslinking studies on Leydig cell mitochondria using metabolically labeled bioactive [^{35}S]DBI *(61)*. Two protein complexes were specifically labeled with in 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. A second complex, migrating at 65 kDa, with an unidentified 55 kDa protein crosslinked to radiolabeled 10 kDa DBI was also formed.

DBI in Steroid/Neurosteroid Synthesis

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 *(95)*. 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 *(96)*, and to have a long-half life *(97)*. We examined the effect of isolated 10 kDa DBI on mitochondria from adrenocortical and Leydig cells *(94)*. Dose-response curves indicated that a threefold stimulation is obtained with low concentrations (0.1–1 μ*M*) of DBI, whereas higher concentrations have lower stimulatory effect on PREG formation. The stimulation obtained was similar to those reported for the 8.2 kDa des-(Gly-Ile)-DBI on bovine adrenocortical mitochondria *(95,98)*. Moreover, similar results were obtained using purified rat and bovine testis DBI *(18)*. In order to exclude the possibility that the stimulatory effect of DBI was owing to the α-helical structure of the protein, we used as control β-endorphin, which also possesses α-helical structures. β-endorphin did not affect the mitochondrial steroid synthesis *(94)*.

As previously noted, high concentrations of DBI (10 μ*M*) gave lower stimulation of steroid synthesis than 100 n*M* DBI. When DBI was added in combination with a maximally stimulating concentration of Ro5-4864 (100 n*M*), the stimulatory effect of Ro5-4864 was abolished, suggesting that in C6-2B glial cells DBI may act as a partial agonist of PBR *(99)*.

We then showed that the amino-acid sequence 17–50 of the DBI bears the biological activity because the triacontatetraneuropeptide (TTN, DBI[17–50]) specifically stimulated mitochondrial steroidogenesis with a potency and efficacy similar to that of DBI *(94)*. 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 *(93)*.

DBI and DBI processing products were also found to be present in brain and C6-2B glioma cell extract. DBI stimulated PREG formation by twofold in mitochondrial fractions from C6-2B glioma cells and rat brain (*55*; Table 2). In addition to DBI, the DBI peptide fragments DBI[17–50] and DBI[39–75] were found to be biological active in in vitro assays *(55,82,84)*, whereas conflicting data has been presented for the fragment octadecaneuropeptide DBI[33-50] *(55,82)*.

Taking into account the findings that

1. hCG increases PBR ligand binding *(57)*;
2. DBI stimulates mitochondrial steroid formation acting via PBR *(61,94)*; and
3. DBI is preferentially localized in the periphery of mitochondria *(100)*, the possibility is raised 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 commonly encountered oligodeoxynucleotide-uptake problems, we took advantage of the ability of steroidogenic cells to utilize exogenous cholesterol via the lipoprotein endocytotic pathway *(101)*. 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' to the initiation codon ATG and 12 downstream the 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. The 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 twofold increase in the amount of steroids produced was observed owing 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 *(101)*.

Using similar technology we also decreased DBI levels in the R2C Leydig cells *(61)*. 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. Because 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 P450scc activity examined in mitoplasts, DBI induced a twofold stimulation of PREG synthesis. Evidence has been previously discussed 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 PREG production by inner mitochondrial membranes implies that this protein can also act via an additional PBR-independent mechanism. Further evidence which indicates the DBI acts directly on P450scc was then provided by observations in an in vitro reconstituted enzyme system *(102,103)*, where DBI stimulated the production of PREG, suggesting that the non-PBR mechanism involved in steroido-

genesis may result from direct activation of P450scc, or alternatively an indirect mechanism that may act via increasing the availability of cholesterol or by altering the rate of reduction of P450scc.

CONCLUSIONS

Considering the data presented in this review, we propose that the steroidogenic pool of cholesterol enters the channel formed by the 18 kD PBR protein and transfers from the outer leaflet of the outer mitochondrial membrane to the inner leaflet of the outer membrane. This activity may be directly stimulated by hormones via modulation of the PBR affinity for the endogenous ligand DBI, which is continuously present around the mitochondria *(100)*. Hormone or cAMP-induced changes in PBR affinity will lead to changes in PBR topography and the rapid formation of contact sites between the outer and inner mitochondrial membranes. Thus, cholesterol will be transported "passively" from the outer to the inner membrane. On the mitochondrial membrane contact-site formation, we should also consider here the role of DBI as an acyl-CoA binding protein *(90)*. DBI was shown to mediate intermembrane transport of long-chain acyl-CoA esters *(104)* and fatty acylation has been proposed as a mechanism employed in transport processes that require fusion of lipid bilayers *(105)*. Thus, DBI may induce the formation of additional contact sites.

It is evident that the presence of tissue-specific PBR-associated proteins may provide selectivity and sensitivity to the PBR function. In addition, it should be noted that the model proposed here does not exclude the presence of additional mechanisms, such as guanosine triphosphate (GTP) and calcium, involved in the process of the acute regulation of steroidogenesis *(46,106,107)*. Identifying and understanding the role of each component of the mitochondrial cholesterol transport apparatus and then their interaction and relationship should allow us to put together the puzzle of the acute regulation of steroidogenesis by hormones. This puzzle is even more complex in the brain because we have no information on the extracellular signal(s) regulating brain neurosteroid synthesis.

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