PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 69 (1999) 123–130

The Journal of
Steroid Biochemistry
&
Molecular Biology

In vitro studies on the role of the peripheral-type benzodiazepine receptor in steroidogenesis ☆

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Abstract

In vitro studies using isolated cells, mitochondria and submitochondrial fractions demonstrated that in steroid synthesizing cells, the peripheral-type benzodiazepine receptor (PBR) is an outer mitochondrial membrane protein, preferentially located in the outer/inner membrane contact sites, involved in the regulation of cholesterol transport from the outer to the inner mitochondrial membrane, the rate-determining step in steroid biosynthesis. Mitochondrial PBR ligand binding characteristics and topography are sensitive to hormone treatment suggesting a role of PBR in the regulation of hormone-mediated steroidogenesis. Targeted disruption of the PBR gene in Leydig cells in vitro resulted in the arrest of cholesterol transport into mitochondria and steroid formation; transfection of the mutant cells with a PBR cDNA rescued steroidogenesis demonstrating an obligatory role for PBR in cholesterol transport. Molecular modeling of PBR suggested that it might function as a channel for cholesterol. This hypothesis was tested in a bacterial system devoid of PBR and cholesterol. Cholesterol uptake and transport by these cells was induced upon PBR expression. Amino acid deletion followed by site-directed mutagenesis studies and expression of mutant PBRs demonstrated the presence in the cytoplasmic carboxy-terminus of the receptor of a cholesterol recognition/interaction amino acid consensus sequence. This amino acid sequence may help for recruiting the cholesterol coming from intracellular sites to the mitochondria. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Eukaryotic steroid hormones, derived from cholesterol, are responsible for the maintenance of the organism's homeostasis, adaptability to the environment and developmental and reproductive functions. Steroidogenesis is regulated by trophic hormones and cAMP which triggers three responses: (i) changes in the state of phosphorylation of specific proteins, (ii) induction of protein synthesis and (iii) stimulation of lipid synthesis [1–4]. One or all of these 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 involving the cytochrome P450 side chain cleavage enzyme (P450scc). The reaction catalyzed by P450scc is not the rate-limiting step in steroidogenesis. Rather, the rate-determining step in the regulation of steroidogenesis and the primary site of acute hormone action is the process of cholesterol delivery to P450scc across the mitochondrial membranes [1–4]. Pregnenolone formed then leaves the mitochondrion to undergo enzymatic transformation in the endoplasmic reticulum that will give raise to the final steroid products.

2. Characterization and localization of the peripheral-type benzodiazepine receptor (PBR)

PBR was originally discovered as another class of

☆ Proceedings of the Xth International Congress on Hormonal Steroids, Quebec City, Quebec, Canada, 17–21 June 1998.

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PII: S0960-0760(99)00056-4

binding sites for benzodiazepines distinct from the central $GABA_A$/benzodiazepine neurotransmitter receptors [5]. Subcellular fractionation studies demonstrated that PBR was primarily localized on mitochondria and more specifically on the outer mitochondrial membrane of steroidogenic cells [6], although a plasma membrane location for this receptor has been identified [7].

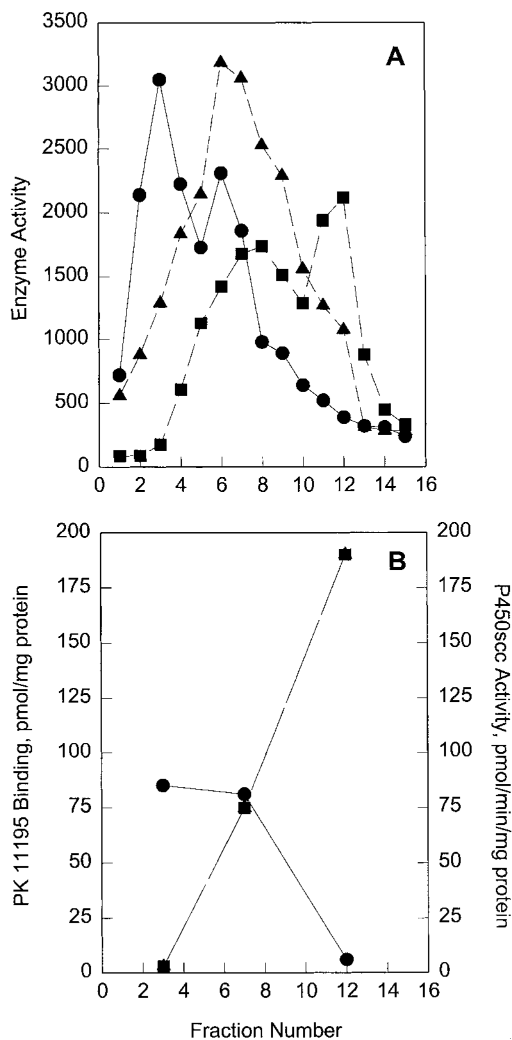

Fig. 1. Separation of submitochondrial membranes by density gradient centrifugation. MA-10 Leydig cell mitochondria were exposed to a swelling, shrinking and sonication procedure as described [47]. Submitochondrial fractions were then centrifuged on a 15-40% sucrose density gradient. The activities of various marker enzymes were determined in the different fractions of the gradient (A). Monoamino oxidase (circles), pmol/min/mg protein, a marker of the outer mitochondrial membrane; Cytochrome c oxidase (squares), nmol/min/mg protein, a marker of the inner mitochondrial membrane; Nucleotide-diphosphate kinase (triangles) (nmol/min/mg protein), a marker of the intermembrane contact sites. (B) PBR ligand binding (circles), B_{max} (pmol/mg protein) and K_d (nM), in each fraction was determined using PK 11195 as the ligand [16]. The ability of the fractions to synthesize pregnenolone (squares) in the presence of cholesterol was measured as described [16,18]. Note that PBR is present in the outer membrane and the contact sites whereas pregnenolone can be formed mostly by the inner membrane and in part by the contact site fraction. Moreover, the K_d of PBR in the outer membrane was 1.6 nM, in the contact sites 0.8 nM and in the inner membrane 2.1 nM indicating that the receptor present in the contact sites fraction has a higher affinity than the outer membrane PBR.

The 18 kDa isoquinoline binding protein was partially purified and the corresponding cDNA was cloned from rat, human, bovine and murine species [8]. The cDNA sequence of the 18 kDa protein specifies an open reading frame of 169 ammo acids, rich in tryptophan residues, with high sequence homology (>80%) across species. Expression studies 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 PBR-associated proteins important for PBR ligand binding expression cannot be excluded. Indeed, we demonstrated that the ability of the 18 kDa PBR protein to bind benzodiazepines was due to its association with 34 kDa voltage-dependent anion channel (VDAC); VDAC by itself does not express any drug binding [9]. This observation and the report that the 18 kDa PBR was isolated as a complex with VDAC and the inner mitochondrial membrane adenine nucleotide carrier [10], suggested that PBR is not a single protein receptor but a multimeric complex. VDAC is a large-conductance ion channel, 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 the mitochondrial permeability transition [11]. Interestingly, PBR was also localized in the mitochondrial contact sites of Leydig cells (Fig. 1).

The topography and organization of PBR was examined in native MA-10 Leydig tumor cell mitochondrial preparations by transmission electron and atomic force microscopy. 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 [12].

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 developed using molecular dynamics simulations [13,14]. According to this model, the five transmembrane domains of PBR were modeled as five α helices, which span one phospholipid bilayer of the outer mitochondrial membrane. This receptor model was 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 (Fig. 2). Considering the PBR complex formation at the contact sites, this cholesterol movement could end in the inner mitochondrial membrane. Thus, this theoretical model supports the role of PBR in cholesterol transport.

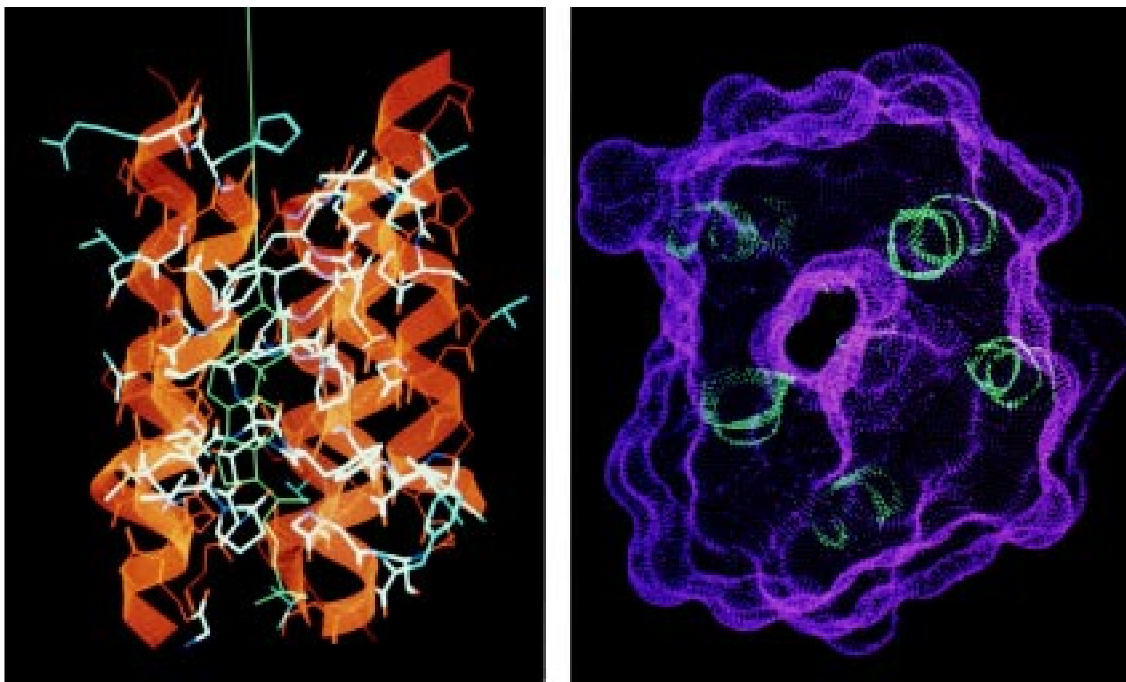

Fig. 2. Molecular modeling of PBR. All calculations were performed with the SYBYL software using the Amber united atom and the tripos all atom force field as previously described for the human receptor [13]. The computer system used was an Indigo 2 of Silicon Graphics. After mutation of the human sequence into the mouse sequence we have constrained the 3D model by an aggregate of the backbone and then submitted it to molecular dynamics in order to eliminate bad contacts of Van der Waals nature between the side chains. We followed this dynamic molecular step with minimization. The aggregate was then removed and the hydrogen bonds were replaced within the helices by C–N harmonic constraints ($r = 2.9$ A, $k = 400$ kcal/mol/A). The dynamic simulation was conducted by a progressive warming up of the system (1 ps at 100 and 200 K), followed by the dynamic simulation for 10 ps at 300 K and cooling at 100 K for 1 ps. At the end of the dynamic simulation the model was minimized by the Powell method with a termination criterion of gradient norm lower than 0.05. The cholesterol molecule was built and minimized using tripos all atom force field. It was then stripped of its hydrogens and its atoms were converted into amber force field types for further calculations in relation to the PBR transmembrane domain. In order to check whether PBR could form a channel allowing cholesterol to cross over the membrane, molecular dynamic simulations were performed using the PBR model into which the cholesterol molecule was pulled by a light constraint, linking the oxygen of the cholesterol molecule to a dummy atom situated at 60 A of the cholesterol and on the other side of the PBR. The force of the harmonic constraint was set to 1 kcal/mol/A. Left: cholesterol molecule (green) in the five-helix bundle, is shown. Right: top view of the PBR five-helix bundle accommodating a cholesterol molecule through the molecular dynamics simulation. The cholesterol molecule can cross the five-helix bundle from one side to the other without serious geometrical modification or energy increase.

3. PBR in steroidogenesis

Because PBR is found primarily on outer mitochondrial membranes and is extremely abundant in steroidogenic cells, we examined the effect of various ligands which bind to PBR on steroid biosynthesis [15,16]. We demonstrated that PBR ligands stimulate pregnenolone production by isolated mitochondria [16]. These data were recently repeated using radiolabeled cholesterol as the substrate ([17] and Fig. 3). This effect on intact mitochondria was not observed with mitochondria devoid of the outer membrane in agreement with the outer mitochondrial membrane localization of the receptor [16]. We also 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 [18]. Thus, the abundance of PBR in steroidogenic tissues together with the tissue-specific cholesterol transport makes PBR a regulator of this rate-determining process. Studies by different laboratories corroborated these observations [19,20] and extended them to ovarian granulosa cells [21]. In addition, a similar mechanism was shown to regulate steroid synthesis in the placenta trophoblasts [22] and brain glial cells [23–26]. Moreover, a PBR-dependent cholesterol transport mechanism was identified in liver mitochondria [27] where this process may be required for cholesterol detoxification from the periphery by the inner mitochondrial membrane sterol-27-hydroxylase. In summary, these data suggest that PBR may be a common mediator of cholesterol transport.

4. PBR endogenous ligands

In addition to the well-characterized PBR drug

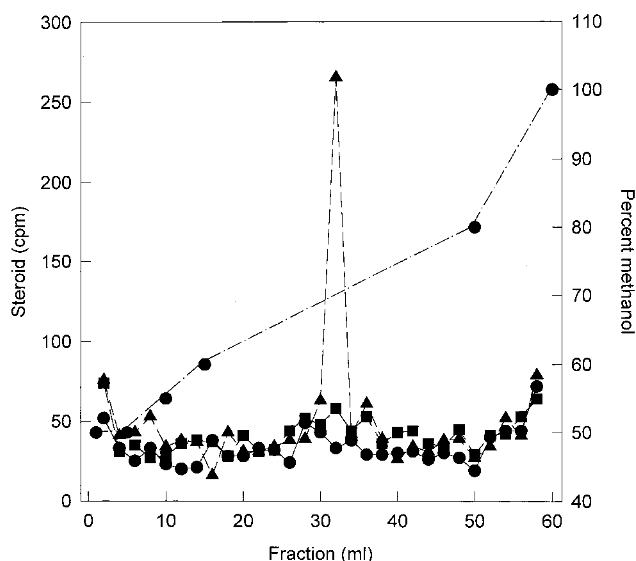

Fig. 3. Effect of Ro5-4864 on MA-10 mitochondrial pregnenolone formation. MA-10 mouse Leydig tumor cells were grown in modified Waymouth's MB752/1 medium and their mitochondria isolated by differential centrifugation as previously described [16,18]. Mitochondria (1.4 mg/ml) were incubated in a buffer containing trilostane (a 3β-hydroxysteroid dehydrogenase inhibitor) and [^{3}H]cholesterol with and without the PBR drug ligand Ro5-4864 (1 μM) and the P450scc inhibitor aminoglutethimide). Samples were incubated for 5 min at 37°C. Then, a mixture of isocitrate and NADP (15 and 0.5 mM final concentrations, respectively) was added to the samples, which were incubated for 4 additional minutes. The reactions were stopped by adding diethyl ether and the steroid extracted with diethyl–ether/ethyl acetate (1:1, v:v). Organic phases were collected, evaporated to dryness and resuspended in methanol. Steroids were separated by HPLC (Beckman System Gold, Fullerton, CA) using a Beckman ultrasphere XL 3 μm Spherical 80 A pore column equilibrated with methanol (50%) in water and eluted at a 1 ml/min flow rate with a 50–100% gradient of methanol. Radioactivity in all fractions was measured by liquid scintillation spectrometry. Steroids were identified by their respective retention time as compared to radiolabeled steroid standards (R_t for pregnenolone = 31 min, R_t for progesterone = 24 min; R_t for DHEA = 18 min, R_t for cholesterol = 67 min). [^{3}H]cholesterol alone (circles); [^{3}H]cholesterol with mitochondria (squares); [^{3}H]cholesterol with mitochondria in the presence of Ro5-4864 (triangles). Circles connected by a broken line indicate the % methanol. It should be noted that aminoglutethimide blocked the effect of Ro-5-4864.

ligands, two other entities were identified as endogenous PBR ligands, porphyrins [28] and the polypeptide diazepam binding inhibitor (DBI) [8,29]. Since in our model system porphyrins were found to have no effect on mitochondrial steroid formation, we focused our studies on the role of DBI, a 10 kDa protein originally purified from brain by monitoring its ability to displace diazepam from the $GABA_A$ receptors [29]. DBI was also independently purified and characterized for its ability to bind long chain acyl-CoA-esters [30]. DBI was found to be highly expressed in steroidogenic cells [29]. DBI displaced radiolabeled benzodiazepines in competition studies and radiolabeled DBI was cross-linked to PBR [31]. Others and we then demonstrated that isolated rat and bovine testis DBI stimulated mitochondrial pregnenolone formation [23,32–34]. We also identified naturally occurring DBI processing products in adrenal, testis and brain extracts. These DBI peptide fragments were biologically active in vitro, displacing PBR drug ligands and stimulating mitochondrial pregnenolone formation [23,32]. The role of DBI in the acute hormone-stimulated steroidogenesis was conclusively shown by suppressing DBI levels in the hormone-responsive MA-10 [35] and the constitutively steroid producing R2C Leydig cells [31], using a cholesterol-linked phosphorothioate oligodeoxynucleotide antisense to DBI. DBI-depleted cells did not produce steroids, suggesting that the presence of DBI is crucial for the acute stimulation of steroidogenesis by trophic hormones and the constitutive steroid synthesis. Taken together these results identify PBR and DBI as common elements of the steroidogenic machinery and strongly imply that PBR is likely to comprise at least part of the mitochondrial intermembrane cholesterol translocation apparatus.

In addition to the in vitro studies presented above, there are numerous in vivo findings that support the role of PBR in steroidogenesis that we recently reviewed [36].

5. Hormones, steroidogenesis and PBR

Flunitrazepam, a benzodiazepine which 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 that was verified as being PBR. 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 P450scc. These observations demonstrated that the antagonistic action of flunitrazepam on hormone-stimulated steroidogenesis is mediated through its interaction with PBR, suggesting that this process involves PBR.

We then examined whether hCG alter the mitochondrial PBR topography using transmission electron and atomic force microscopy of immunolabeled, with anti-PBR, mitochondrial membranes [38]. Acute treatment with hCG induced the appearance of large clusters varying from 15 to 25 gold particles, or more, in contrast to the 4–6 particle clusters present in mitochondria from control cells. Atomic force microscopic analysis of these areas further demonstrated the reorganization of the mitochondrial membrane. The specificity of the effect of hCG was determined by treating cells with hCG and H-89, a selective inhibitor of PKA,

shown to block the hormone-induced PBR binding and steroid formation [38]. The inhibitor also blocked the effect of hCG on PBR topography. In addition, flunitrazepam also blocked the effect of hCG on PBR distribution on mitochondrial membranes. Under the same conditions hCG treatment resulted in a very rapid increase in PBR binding capacity (within a min). This increase gradually returned to basal levels. Scatchard analysis revealed that in addition to the known high nM affinity benzodiazepine binding site, a second higher affinity (0.2 nM), hormone-induced, benzodiazepine binding site appeared [39]. Under the same conditions, hCG increased the rate of mitochondrial pregnenolone formation. H-89 blocked both the hormone-induced PBR binding and steroid formation. Furthermore, flunitrazepam abolished the hCG-induced rapid stimulation of steroid synthesis. These results demonstrate that, in Leydig cells hCG and cAMP rapidly induce the reorganization of mitochondrial membranes favoring the formation of contact sites that may facilitate the intramitochondrial cholesterol transfer. Thus, free cholesterol from the outer mitochondrial membrane would go via the contact sites to the inner membrane P450scc. This change in topography will result in the transient induction of a higher affinity benzodiazepine binding site which occurs concomitantly with the increase in the rate of steroid formation [39], suggesting that hormones alter PBR to activate cholesterol delivery to the inner mitochondrial membrane and subsequent steroid formation.

6. Steroidogenesis in PBR knock-out Leydig cells

R2C cells are derived from rat Leydig tumors and maintained their in vitro capacity to synthesize steroids constitutively in a hormone-independent manner [40]. Radioligand binding assays on intact Ra2C cells revealed the presence of a single class of PBR binding sites with an affinity 10-times higher than that displayed by the MA-10 PBR [31]. Photolabeling of R2C 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 which has properties similar to the MA-10 PBR. Moreover, a PBR ligand was able to increase steroid production in mitochondria from R2C cells. Interestingly, mitochondrial PBR binding was increased by 6-fold upon addition of the postmitochondrial fraction prepared from cells expressing the higher affinity receptor, suggesting that a cytosolic factor modulates the binding properties of PBR in R2C cells and is responsible for the higher affinity receptor seen in intact cells [31]. The presence of a cytosolic steroidogenesis-stimulating factor in R2C cells and the constitutive expression of StAR by these cells have been reported [40,41]. These data demonstrate that ligand binding to the mitochondrial higher affinity PBR is involved in maintaining R2C constitutive steroidogenesis and that a cytosolic factor may be involved in the regulation of this process.

To provide conclusive evidence about the role of PBR in steroidogenesis, we developed a molecular approach based on the targeted disruption of PBR gene in R2C rat Leydig cell line by homologous recombination [42]. On the basis of the known rat PBR gene sequence, we designed a targeting vector containing the *neo* gene, conferring the neomycin resistance which allows for a positive selection of cells that have undergo homologous recombination, in between the two PBR genomic DNA fragments and 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. A G418/Ganciclovir-resistant cell line was generated. PBR expression, examined by ligand binding, immunoblot and immunoelectron microscopy analyses, was absent in this cell line. In addition, the PBR-negative R2C(-) cells produced minimal amounts of progesterone compared to normal R2C cells. Addition of the hydrosoluble derivative of cholesterol 22*R*-hydroxycholesterol, or transfection of the cells with a PBR cDNA, rescued the 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 presence of the StAR protein in R2C cells has been shown and it has been postulated that constitutive steroidogenesis in these cells is maintained by the constitutive expression of StAR in these cells [41]. We examined the presence of StAR in these cells. Surprisingly, StAR levels remained the same in control and PBR negative cells where steroidogenesis ceased, suggesting that StAR may act before or at the level of PBR to regulate steroid production.

In agreement with our findings, Gavish and coworkers recently reported that transfection of MA-10 cells with an antisense knock out vector, working under the cAMP-dependent enkephalin promotor, decreased PK 11195 ligand binding capacity by 40% [43]. Treatment of the cells with cAMP for 12 h to induce the expression of the antisense vector resulted in the decrease of cAMP-dependent progesterone production by 40%, further demonstrating the direct correlation between PBR expression and cAMP-dependent steroidogenesis.

7. Gain of function studies in bacteria

Bacteria do not have cholesterol and PBR protein

or ligand binding. *Escherichia coli* were transfected with mouse PBR cDNA. Addition of isopropyl-1-thiol-β-D-galactopyranoside (IPTG) to transfected bacteria induced the expression of the 18 kDa PBR protein and ligand binding with similar pharmacological characteristics to that previously described for PBR. IPTG-induced PBR expression resulted in a protein, time and temperature-dependent uptake of radiolabeled cholesterol [17]. No uptake of other radiolabeled steroid could be seen. When IPTG-induced, cholesterol-loaded, bacterial membranes where 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 [44]. Thus, a tissue and cell specific cholesterol homeostasis is achieved. Considering the widespread occurrence of PBR and its tissue and cell specific subcellular localization, these results suggest a more general role for PBR as a cholesterol channel in intracellular cholesterol transport and compartmentalization.

Recently, we performed a series of deletions in order to define the ligand binding and cholesterol binding domains of the 18 kDa PBR protein. The truncated forms of PBR, PBRΔ5–20 and PBRΔ41–51 expressed reduced ability to bind drug ligands, although they kept the ability to uptake cholesterol. The carboxy-terminal truncated PBRΔ153–169 could not uptake cholesterol although it retained full capacity to bind drug ligands. Site-directed mutagenesis identified two amino acids interacting with cholesterol, Y153 and R156, because bacteria expressing the mutant PBR proteins PBR(Y153S) and PBR(R156L) do not accumulate cholesterol. Considering these results we postulated the existence of a common cholesterol recognition/interaction amino acid consensus pattern – L/V– $(X)_{1-5}$–Y– $(X)_{1-5}$–R/K– (CRAC). Indeed, we found this amino acid consensus pattern in all proteins shown to interact with cholesterol [17]. In conclusion, these data suggest that the expression of PBR confers the ability to take up and release, upon ligand activation, cholesterol. In addition, these results also suggest that PBR has two distinct cholesterol and ligand binding sites. Considering that proteins in the outer mitochondrial membrane are positioned with the carboxy terminal domain at the cytoplasmic side together with the recent finding that the PBR α-helices are sufficiently long to span the entire membrane bilayer [45], then a model for PBR-mediated cholesterol uptake was developed (Fig. 4).

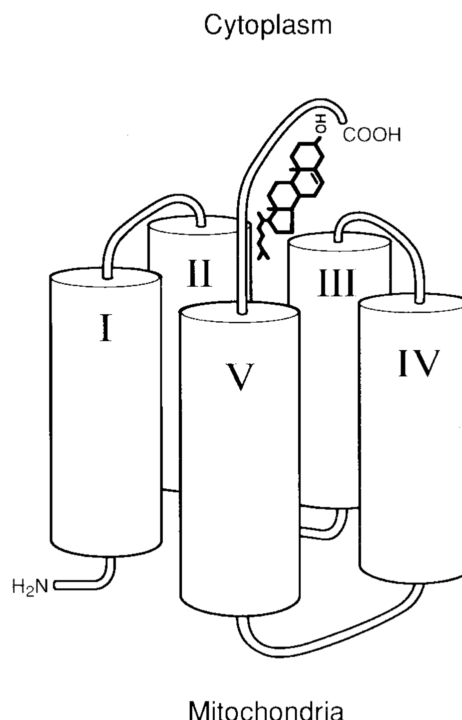

Fig. 4. Model of cholesterol uptake by PBR based on data generated by deletion analysis. Cholesterol is coming from the cytoplasm probably bound to a protein, such as sterol carrier protein-2 or StAR. The exchange of cholesterol takes place at the outer mitochondrial membrane where cholesterol binds to the carboxy terminus of the receptor, which then brings the steroid in the channel. I, II, III, IV and V represent the five transmembrane domains of the receptor.

8. Novel approaches to study PBR function

Based on the structure of high affinity drug ligands for PBR, a 7-nitro-2,1,3-benzoxadiazol-4-yl derivative of 2-phenylindole-3-acetamide (compound **4**) was synthesized [46]. This fluorescent probe displaced the radiolabeled PK 11195 with a K_i of 10 nM. 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 to probe the localization and function of PBR in living cells, in

different tissues and under conditions where antisera cannot penetrate.

Recenlty, using the yeast two hybrid system and PBR as the bait, we screened a mouse testis cDNA library in order to identify PBR-associated proteins (PAPs). Using this method, we isolated the cDNAs for 5 PAPs (Li and Papadopoulos, unpublished). The characterization and interaction of these proteins with PBR is under investigation. Preliminary data indicates that these proteins may be part of the PBR activation mechanism responsible for the hormonal induction of the 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 mediate a StAR-PBR interaction responsible for the continuous supply of the steroidogenic machinery with cholesterol that, through PBR, will reach the P450scc.

9. Conclusions

PBR seems to serve the function of cholesterol channel in different tissues and across species. In specialized tissues such as adrenals, gonads, placenta and brain, this function is associated with increased steroid synthesis. The fact that PBR plays a role in steroidogenesis is suggested by the following observations: (1) PBR is highly expressed in steroidogenic tissues where it is primarily localized in the outer mitochondrial membrane and contact sites; (2) its affinity and topography is regulated by peptide trophic hormones; (3) PBR ligands stimulate mitochondrial cholesterol transport and steroid synthesis; (4) PBR knock out Leydig cells do not synthesize steroids but this function is rescued by transfecting the cells with the PBR cDNA and (5) PBR expression in a PBR and cholesterol devoid system confers to these cells the ability to uptake and transport cholesterol. Although steroidogenesis is a hormone-regulated process in adrenals and gonads, this process seems to be constitutive and/or developmentally regulated in placenta and brain. Alternatively, a common third messenger system may exist in all steroidogenic tissues, which responds to tissue-specific second messenger systems. 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 factors, resulting in cholesterol movement across the mitochondrial membranes. In this scheme, PBR is the 'gate keeper' controlling the rate of steroid synthesis.

Acknowledgements

This work was supported by grants R01-ES-07747 from the National Institute of Environmental Health Sciences and K04-HD-01031 from the National Institute of Child Health and Human Development, NIH (to VP).

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