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Role of the Peripheral-type Benzodiazepine Receptor and the Polypeptide Diazepam Binding Inhibitor in Steroidogenesis

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Steroidogenesis begins with the metabolism of cholesterol to pregnenolone by the inner mitochondrial membrane cytochrome *P*450 side-chain cleavage (*P*450scc) enzyme. The rate of steroid formation, however, depends on the rate of (i) cholesterol transport from intracellular stores to the inner mitochondrial membrane and (ii) loading of *P*450scc with cholesterol. We demonstrated that a key element in the regulation of cholesterol transport is the mitochondrial peripheral-type benzodiazepine receptor (PBR) and that the presence of the polypeptide diazepam binding inhibitor (DBI) was vital for steroidogenesis. We also showed that DBI, as the endogenous PBR ligand, stimulates cholesterol transport. In addition, DBI directly promotes loading of cholesterol to *P*450scc. We review herein our studies on the structure, function, topography and hormonal regulation of PBR and DBI in steroidogenic cells. Based on these data we propose a model where the interaction of DBI with PBR, at the outer/inner membrane contact sites, is the signal transducer of hormone-stimulated and constitutive steroidogenesis at the mitochondrial level. Hormone-induced changes in PBR microenvironment/structure regulate the affinity of the receptor. PBR ligand binding to a higher affinity receptor results in increased cholesterol transport. In addition, hormone-induced release (processing?) of a 30,000 M_W DBI-immunoreactive protein from the inner mitochondrial membrane may result to the intramitochondrial production of DBI which directly stimulates loading of *P*450scc with cholesterol. Thus, *in vivo*, hormonal activation of these two mechanisms results in efficient cholesterol delivery and utilization and thus high levels of steroid synthesis.

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INTRODUCTION

Trophic hormone regulation of steroid synthesis can be thought as being either "acute", that occurs within minutes and results in the rapid synthesis of steroids, or "chronic", that occurs over a long period of time and results in increased synthesis of the components of the steroidogenic machinery and 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 cholesterol is converted to pregnenolone by the cholesterol side chain cleavage cytochrome *P*450 (*P*450scc) and auxiliary electron transferring proteins, localized on inner mitochondrial membranes [1–4]. 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 *P*450scc active site [1–4]. 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 [1–4].

DBI IN STEROIDOGENESIS

In search of the cytoplasmic steroidogenesis-stimulating factor(s), a protein of molecular weight (M_W) 8200, was isolated from bovine adrenals [5, 6]. This protein stimulates transport of cholesterol into

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mitochondria and transport from the outer to the inner membrane [5, 6]. This 8200 steroidogenesis-stimulating protein was shown to be identical to the polypeptide diazepam binding inhibitor (DBI) [7], except the loss of two amino acids (Gly-Ile) from the carboxy terminus [8]. DBI, a 10,000 M_W protein, was originally purified from brain by monitoring its ability to displace diazepam from the allosteric modulatory sites for τ-aminobutyric acid (GABA) action on $GABA_A$ receptors [7, 9]. DBI was also independently purified and characterized for its ability to bind long chain acyl-CoA-esters [10] and modulate insulin secretion [11]. DBI was found to be present in a variety of tissues and to be highly expressed in adrenocortical and testicular Leydig cells [12–15]. We and others then showed that des-(Gly-Ile)-DBI, purified rat and bovine brain, rat testis and bovine testis DBI stimulate intramitochondrial cholesterol transport and increase pregnenolone formation by isolated adrenocortical and Leydig cell mitochondria [3, 6, 8, 16, 17]. The bioactive amino acid sequence within the DBI molecule bearing the steroidogenesis-stimulating activity was identified in the region of amino acids 17–50 [16]. Moreover, we showed that both native and recombinant DBI were able to stimulate loading of the *P*450scc with cholesterol in an *in vitro* reconstituted enzyme system [18, 19]. The significance of this finding will be discussed later in the manuscript.

Although DBI was initially thought to be a short lived protein, regulated by hormones and cAMP [6], we demonstrated that it has a long half life and it is not regulated by hormones in steroidogenic cells [13]. However, the presence of DBI was shown to be vital for the acute trophic hormone-stimulation of steroidogenesis since suppression of DBI expression in MA-10 Leydig cells, using a cholesterol-linked phosphorothioate oligodeoxynucleotide antisense to DBI, resulted in inhibition of hormonal stimulation [20]. These data suggest that DBI may act on mitochondrial pregnenolone formation through a hormone-regulated mechanism.

The role of DBI in a cell model where steroidogenesis occurs in a hormone-independent, constitutive, manner was also examined [21]. In these cells, 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. The R2C rat Leydig constitutive steroid producing tumor cells expressed high levels of DBI. R2C cell treatment with a cholesterol-linked phosphorothioate oligodeoxynucleotide antisense to DBI, but not sense, resulted in the reduction of DBI levels and a concomitant dramatic decrease of the amount of progesterone produced [21]. These observations strongly suggest that DBI was also important in maintaining constitutive steroidogenesis.

PBRs IN STEROIDOGENESIS

PBRs were originally discovered as a binding site for the benzodiazepine diazepam in peripheral tissues [22, 23]. The reason this drug receptor attracted much interest is that benzodiazepines are among the most highly prescribed drugs due to their pharmacological actions in relieving anxiety mediated through modulating the activity of GABA receptors in the central nervous system. Reviews on the biochemistry and pharmacology of the PBR were recently published [22, 23].

Two important observations indicated that PBRs are likely to play a role in steroidogenesis: (1) PBRs are found primarily on outer mitochondrial membranes [22, 23]; and (2) we and others showed that PBRs are extremely abundant in steroidogenic cells [23]. Moreover, we reported the use of a spectrum of ligands which bind to PBR and demonstrated that these drugs, via binding to PBR, stimulate steroid biosynthesis in several adrenocortical and Leydig cell systems [24, 25]. This effect was not additive to the stimulation by hormones and cAMP [26]. Moreover, we demonstrated that PBR ligands stimulated pregnenolone biosynthesis by isolated mitochondria, in a dose-dependent manner. This effect was not observed with mitoplasts (mitochondria devoid of the outer membrane). In subsequent studies we verified that PBR did not act by directly stimulating *P*450scc [25]. Similar results were also obtained using bovine and rat adrenocortical cells as well as purified rat Leydig cells and brain glial cells [24–27]. In these studies we concluded that PBRs 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. More recently, we provided evidence that PBR ligand-induced stimulation of pregnenolone formation was due to PBR-mediated translocation of cholesterol from the outer to the inner mitochondrial membrane [26]. Studies by different laboratories confirmed these observations [17, 28] and extended them to placental [29] and ovarian granulosa cells [30].

In contrast to these observations, high micromolar concentrations of benzodiazepines were found to inhibit aldosterone and ACTH-induced corticosterone synthesis by adrenal cells [31, 32]. Examining the effects of benzodiazepine PBR ligands on steroidogenesis we have to consider the high affinity, low nanomolar, form of the receptor. Thus, these inhibitory effects may not involve PBR and may represent pharmacologic effects. Indeed, in recent studies, it was found that the inhibitory effect of high concentrations of benzodiazepines was due to the inhibition of microsomal hydroxylases [33] and calcium channels [34]. In addition, when low concentrations of benzodiazepines close to the affinity of PBR were used, the same authors observed stimulation of both aldosterone and

corticosterone synthesis [35]. Thus, consensus is now evident in that a common PBR pathway is functionally linked to steroidogenesis in all steroid synthesizing tissues.

As discussed above, we have shown that PBR mediates cholesterol translocation in mitochondria which accounts for the stimulation of steroidogenesis by PBR ligands in cells and isolated mitochondria [17, 24–30]. However, it was still unclear whether PBRs participated in hormone-stimulated steroidogenesis directly or indirectly. We then reported that flunitrazepam, a benzodiazepine, which binds to PBR with high nanomolar affinity, inhibited hormone- and cAMP-stimulated steroidogenesis [36]. Scatchard analysis 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 pregnenolone formation in isolated mitochondrial preparations which was determined to result from a reduction of cholesterol transport to inner mitochondrial membranes. These observations provided evidence that the antagonistic action of flunitrazepam on hormone-stimulated steroidogenesis is mediated through its interaction with PBR. This conclusion therefore implies that hormone-induced steroidogenesis involves, at least in part, the participation of PBR.

In a more recent study we examined whether PBR is the site of hormone action [37]. Within 15 s of the addition of hCG to MA-10 Leydig cells a 3-fold, cAMP-dependent, increase of PBR binding was observed. This rapid increase returned to basal levels within 60 s. Scatchard analysis revealed that in addition to the known high affinity (5.0 nM) benzodiazepine binding site, a second higher affinity (0.2 nM), 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 cholesterol metabolism, 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 (PKA) blocked not only the hormone-induced PBR binding but also steroid formation. Furthermore, addition of flunitrazepam completely abolished the hCG-induced rapid stimulation of steroid synthesis. These results demonstrate that, in Leydig cells, the most rapid effect described thus far 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. This, in turn, suggests that these hormones alter PBR to activate cholesterol delivery to the inner mitochondrial membrane and subsequent steroid formation.

We also examined the characteristics of PBR in the R2C constitutive steroid producing Leydig cell line [21]. Radioligand binding assays on intact cells revealed the presence of a single class of PBR binding sites with an affinity 10-times higher ($K_d = 0.5$ nM) than that displayed by the MA-10 PBR ($K_d = 5$ nM). Photolabeling of R2C and MA-10 cell mitochondria with a photoactivatable PBR ligand showed that the 18,000 M_W 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 synthetic ligand was able to increase steroid production in isolated mitochondria from R2C cells which express the 5 nM affinity receptor. Interestingly, mitochondrial PBR binding was increased by 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 nM affinity receptor seen in intact cells. In conclusion, these data demonstrate that ligand binding to the mitochondrial higher affinity PBR is involved in maintaining R2C constitutive steroidogenesis.

The state of phosphorylation of PBR *in vitro* and *in situ* was then examined as a possible mechanism of modulation of PBR by hormones [38]. Analysis of the amino acid sequence of the rat [39] and MA-10 [40] PBR, revealed the presence of several putative phosphorylation motifs for cAMP-, Ca^{2+}/calmodulin-, and Ca^{2+}/phospholipid-dependent protein kinases. Interestingly, these kinases have been shown to be involved in the regulation of steroidogenesis. In rat and bovine adrenal mitochondrial preparations PKA, but not other purified protein kinases, was found to phosphorylate the 18,000 PBR protein. In order to examine the *in situ* phosphorylation of the 18,000 PBR protein we used digitonin-permeabilized Leydig cells and [τ-^{32}P]ATP. Under these conditions the phosphorylation of the 18,000 PBR protein was stimulated by cAMP [38]. Thus, these data suggested that PBR is an *in vitro* and *in situ* substrate of PKA. However, cloning of the human 18,000 M_W PBR protein [41, 42] predicted an amino acid sequence missing the phosphorylation motif identified in the rat, mouse, and bovine sequence, thus suggesting that phosphorylation of the 18,000 PBR protein may not be an ubiquitous mechanism of regulation of PBR function.

Considering the above findings on the role of PBR in the regulation of steroidogenesis we proceeded to better characterize the structure of the mitochondrial Leydig cell PBR. PBR was identified and characterized originally by its high affinity for two distinct classes of compounds, the benzodiazepines and the isoquinolines [22, 23]. An 18,000 M_W isoquinoline binding protein has been identified as PBR [22, 23]. We isolated and sequenced a 626 bp cDNA, specifying an open reading frame of 169 amino acid residues with a predicted M_W of 18,843, from MA-10 Leydig cells [40]. Expression of PBR 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 18,000 M_W PBR protein alone or to other constitutively expressed components of the receptor, an *in vitro* system was developed using recombinant PBR protein [40]. Isolated maltose binding protein (MBP)-PBR recombinant fusion protein incorporated into liposomes, but not MBP alone, was able to bind isoquinolines but not benzodiazepines. Addition of MA-10 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 34,000 M_W voltage-dependent anion channel (VDAC) protein, which by itself does not express any drug binding. In addition to these findings, the observation that the 18,000 PBR is structurally [43] associated with the 34,000 M_W VDAC provides strong evidence that PBR is not a single protein receptor but a multimeric complex where the isoquinoline binding site is on the 18,000 M_W subunit and expression of the benzodiazepine binding site requires both the 18,000 and the 34,000 M_W VDAC subunits.

Native MA-10 mouse Leydig tumor cell mitochondrial preparations were then examined by transmission electron and atomic force microscopic 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 indicate that the 18,000 M_W PBR protein is organized in clusters of 4–6 molecules [44]. Moreover, in many occasions, the inter-relationship among the PBR molecules was found to favor the formation of a single pore. Since the 18,000 M_W PBR protein is functionally associated with the pore forming 34,000 M_W 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. Thus, cholesterol and molecules of $M_W < 6000$ [45], could be translocated via the pores formed by PBR/VDAC to the inner mitochondrial membrane where cholesterol will be metabolized by the *P*450scc enzyme to pregnenolone. Interestingly, a three dimensional model of the mitochondrial PBR was recently developed using molecular dynamics simulations [46]. 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 then tested as a cholesterol carrier and it was shown that indeed PBR can accommodate a cholesterol molecule and function as a channel [46]. These authors 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/VDAC association 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 the intramitochondrial cholesterol transport. In addition, our findings on the topography of the receptor suggest that the native receptor is a multimeric complex approximating 140,000 M_W composed of an average of five 18,000 PBR subunits, one 34,000 VDAC subunit, and associated lipids. This value is close to that reported for the digitonin solubilized, photolabeled PBR migrating as a 170,000 apparent M_W protein on gel filtration columns [23].

THE DBI–PBR INTERACTION

All these results taken together identify PBR and DBI as common elements of the steroidogenic machinery in all steroidogenic tissues, in all species examined. However, direct evidence on the interaction of DBI with PBR was still lacking. In order to address this gap we took three distinct experimental approaches to identify DBI as the endogenous ligand of PBR: (i) radioligand displacement studies; (ii) competitive studies with funitrazepam; and (iii) chemical crosslinking studies.

Binding of DBI to PBR was initially determined by examining its ability to displace high affinity radiolabeled PBR drug ligands [7, 10, 12, 14]. Typical competition studies for specific binding of radiolabeled benzodiazepines indicated that DBI displaced benzodiazepines with an inhibitory constant of 1–2 μM. In subsequent studies we analyzed the binding of DBI to PBR by performing similar experiments under conditions identical to those used to study DBI effects on mitochondrial steroid synthesis. We found that the inhibitory constant of DBI for PBR binding inhibition was 100 nM, a value that correlates well with the EC_{50} of DBI for mitochondrial steroid biosynthesis induction [27]. In addition, the stimulatory effect of DBI on mitochondrial pregnenolone formation was specifically blocked by flunitrazepam, the benzodiazepine PBR ligand shown to inhibit hormone-stimulated steroidogenesis [16].

To further demonstrate that DBI specifically binds to PBR, we performed crosslinking studies on Leydig cell mitochondria [21]. Initial studies using DBI radiolabeled *in vitro* with ^{125}I failed because ^{125}I-DBI was found to be inactive. Thus, we prepared metabolically labeled bioactive [^{35}S]DBI [21]. Two protein complexes were specifically labeled with [^{35}S]DBI in R2C Leydig cells [21] as well as in MA-10 Leydig and Y-1 adrenocortical cells (unpublished). A protein complex with an apparent M_W of 27,000 was recognized by an antiserum against PBR, suggesting that the 10,000 M_W DBI formed a specific complex with the 18,000 M_W PBR protein. A second complex, migrating at 65,000 M_W, with an unidentified 55,000 M_W protein crosslinked to radiolabeled 10,000 M_W DBI was also formed. The role of this 55,000 M_W protein in the DBI–PBR-mediated activation of mitochondrial

steroid synthesis should also be considered as well as the possibility that the 55,000 M_W protein could be a PBR trimer or a dimer of the 27,000 M_W complex not recognized by the antisera used.

In conclusion, the observations that PBR drug ligands and DBI bind to PBR, stimulate mitochondrial pregnenolone formation, and can be crosslinked to the 18,000 M_W PBR protein demonstrate the functional significance of DBI–PBR interaction in the stimulation of intramitochondrial cholesterol transport and subsequent steroid formation.

30,000 M_W DBI-IMMUNOREACTIVE PROTEIN AND STEROIDOGENESIS

As noted above, PBR drug ligands did not have any direct effect on *P*450scc activity when examined in mitoplasts [25]. However, when preparations of adrenocortical mitochondrial inner membranes were incubated with DBI, a stimulation of the production of pregnenolone was observed. Evidence has been already discussed which 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 which indicates the DBI acts directly on the steroidogenic enzyme *P*450scc was then provided by observations in an *in vitro* reconstituted *P*450scc enzyme system [18, 19]. In this system, purified *P*450scc enzyme, appropriate electron donors and electron transport proteins were incubated with a source of exogenous cholesterol in the presence or absence of DBI and the benzodiazepine Ro5-4864. Only DBI stimulated the production of pregnenolone. When considered together, these data suggest 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.

These possibilities were investigated further by examining the effect of DBI on the substrate-induced difference spectrum for *P*450scc (unpublished). This technique exploits a property common to all the cytochrome *P*450 enzymes whereby the Soret peak (420 nm) shifts to a different absorbance maxima (around 390 nm) when substrate is bound to the active site. The property is a characteristic of the heme moiety of the *P*450s and indicates a shift from the low spin state to the high spin state of the heme iron [47]. The shift can be measured spectrophotometrically and the difference in absorbance maxima is directly proportional to the proportion of molecules of *P*450 with substrate bound to the active site [48].

When cholesterol is added to a solution containing purified *P*450scc and no electron carriers, a shift in the Soret peak is observed. The magnitude of the shift increases with concentrations of added cholesterol as previously reported [49, 50]. However, we found that for a fixed concentration of cholesterol, addition of DBI causes a further increase in the amplitude of the difference spectrum. This increase is also dependent on the concentration of DBI added over the range 0.5–8 nM. Furthermore, when we measured the rate of reduction of *P*450 with electron carriers and electron donors present, we found it to be 50% greater in the presence of DBI compared to that found in the presence of substrate alone (unpublished).

Since the effect on the substrate induced difference spectrum occurs in the absence of both electron carriers and electron donors, it appears that this effect is caused by direct interaction of DBI with the *P*450scc enzyme. Furthermore, as the magnitude of the Soret peak shift is proportional to the proportion of *P*450 molecules with substrate bound at the active site, we must conclude that DBI increases the loading of substrate (cholesterol) into the *P*450scc active site.

In an attempt to identify a mechanism by which DBI may be active in the regulation of *P*450scc activity, we examined the distribution of DBI within the mitochondria. Using anti-DBI antibodies we identified the 10,000 M_W DBI in the outer mitochondrial membranes of adrenocortical cells but not in the inner membranes [51]. However, in inner mitochondrial membrane preparations, we did observe a 30,000 M_W DBI-immunoreactive protein of 6.8 isoelectric point. Attempts to solubilize this protein indicated that it is deeply embedded within the inner mitochondrial membrane. Moreover, when adrenocortical cells were treated with ACTH for 30 min prior to the preparation of mitochondria, the levels of this 30,000 M_W protein within the inner mitochondrial membrane were reduced by approx. 70% [51]. Due to the immunological homology of this protein with DBI, it is tempting to speculate that the 30,000 M_W DBI-related peptide may be a higher molecular weight form of DBI which is processed by a hormone-dependent mechanism to release DBI directly at the inner mitochondrial membrane.

PROPOSED MODEL

These data together suggest that PBR are involved in the transport of cholesterol from the cytoplasm to within the mitochondria. This activity is stimulated by hormones directly via modulation of the PBR affinity which may also lead to the rapid formation of contact sites between the outer and inner mitochondrial membranes. The endogenous PBR ligand, DBI, which is continuously present around the mitochondria [52], will now bind to PBR and cholesterol will be transported from the outer to the inner membrane within the contact sites. On the mitochondrial membrane contact site formation we should also consider here the role of DBI as an acyl-CoA binding protein [10]. DBI

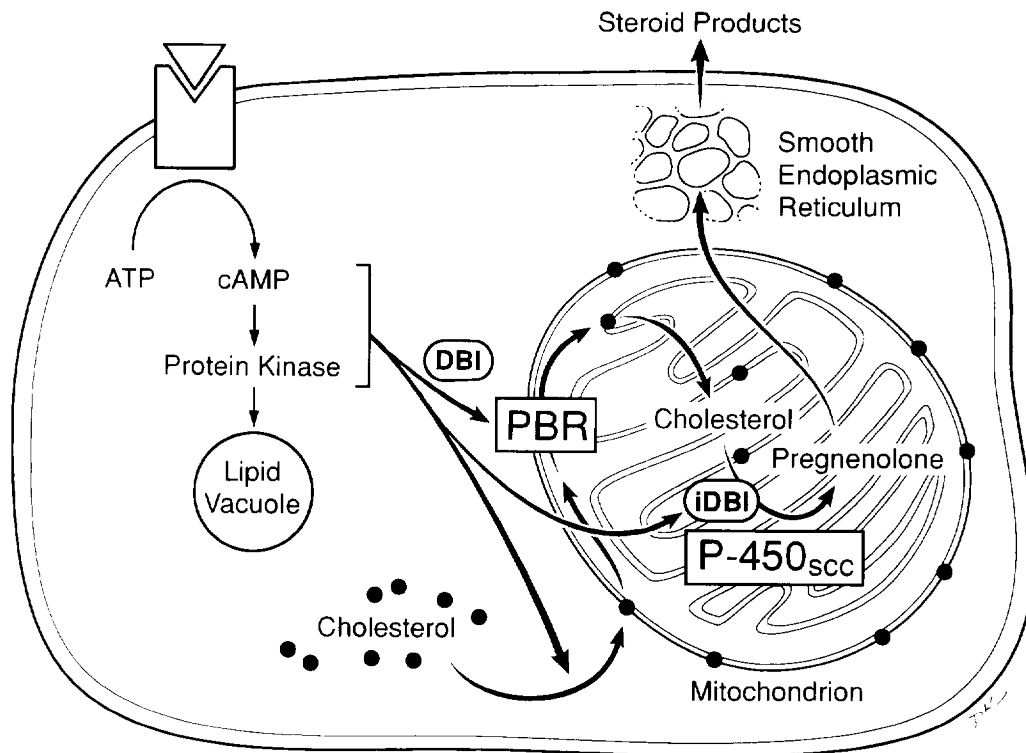

Fig. 1. Schematic representation of the role of DBI and PBR in steroid biosynthesis. Polypeptide hormones interact with a cell surface receptor stimulating cAMP production. Activation of the cAMP-dependent protein kinase results in the liberation of free cholesterol from different intracellular stores. This cholesterol is then transported to the mitochondrion and is incorporated into the outer mitochondrial membrane. PBR plays a role in the incorporation of the cholesterol in the outer membrane and mediates the rate-limiting step in steroid biosynthesis which is transport of cholesterol from outer to inner mitochondrial membranes. This process is activated by the binding of the cytroplasmic DBI to PBR and it may occur at the mitochondrial membrane contact sites. Once associated with the inner mitochondrial membrane cholesterol is accessible to the *P*450scc. The inner mitochondrial membrane 30,000 M_W DBI-immunoreactive protein (iDBI) is then released (proteolysed?) in response to hormones and it increases loading of cholesterol to the *P*450scc, resulting in high levels of pregnenolone formation.

was recently shown to mediate intermembrane transport of long-chain acyl-CoA esters [53] and fatty acylation has been proposed as a mechanism employed in transport processes that require fusion of lipid bilayers [54]. Hence, a further activity of DBI may yet prove to be the "priming" of the mitochondria for steroidogenesis by formation of additional contact sites.

The data obtained with the constitutive steroid-producing R2C Leydig cells, which contained only the higher affinity benzodiazepine binding site along with DBI, further supports the proposed model. Thus, the result of such PBR-mediated action would be to increase the amount of substrate, cholesterol, available to *P*450scc.

If the 30,000 M_W DBI immunoreactive protein responds to ACTH by releasing a DBI fragment, within or near the inner mitochondrial membrane, the *P*450scc enzyme may be "primed" to take advantage of the increased availability of steroidogenic substrate and catalyze its conversion to pregnenolone at a greater rate. This may also explain why we and others see only 2–3-fold increase in steroidogenesis by isolated mitochondria, since no *in vitro* system will ever stimulate both mechanisms simultaneously. However, *in vivo* the stimulation will be amplified by the generation of DBI, from the 30,000 M_W precursor, which increases loading of cholesterol to the *P*450scc. A schematic representation of this model is shown in Fig. 1.

This proposed model does not exclude the presence of additional mechanisms involved in the process of steroidogenesis [55–58]. It is possible that the hormone-induced increase in PBR binding and increase in contact site formation triggers and/or represents the first in a series of events which will sustain steroid synthesis for many hours, e.g. translocation of the 28,000 M_W protein, shown to parallel steroid synthesis [57, 58], from the cytoplasm to the mitochondrion. Better understanding of the relationship between all components involved in the regulation of cholesterol transport should unveil the molecular mechanism(s) mediating hormonal "acute" stimulation of steroidogenesis.

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REFERENCES

1. Simpson E. R. and Waterman M. R.: Regulation by ACTH of steroid hormone biosynthesis in the adrenal cortex. *Can. J. Biochem. Cell. Biol.* **61** (1983) 692–707.
2. Hall P. F.: Trophic stimulation of steroidogenesis: in search of the elusive trigger. *Rec. Prog. Horm. Res.* **41** (1985) 1–39.
3. Kimura T.: Transduction of ACTH signal from plasma membrane to mitochondria in adrenocortical steroidogenesis. Effects of peptide, phospholipid, and calcium. *J. Steroid Biochem.* **25** (1986) 711–716.
4. Orme-Johnson N. B.: Distinctive properties of adrenal cortex mitochondria. *Biochim. Biophys. Acta* **1020** (1990) 213–231.
5. Hall P. F., Papadopoulos V. and Yanagibashi K.: On the mechanism of action of ACTH. In *Progress in Endocrinology* (Edited by K. Shizume and S. Yoshida). Elsevier Science Publ., Amsterdam, Netherlands (1988) pp. 253–258.
6. Yanagibashi K., Ohno Y., Kawamura M. and Hall P. F.: The regulation of intracellular transport of cholesterol in bovine adrenal cells: purification of a novel protein. *Endocrinology* **123** (1988) 2075–2082.
7. Guidotti A., Forchetti C. M., Corda M. G., Konkel D., Bennet C. D. and Costa E.: Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. *Proc. Natn. Acad. Sci. U.S.A.* **80** (1983) 3531–3533.
8. Besman M. J., Yanagibashi K., Lee T. D., Kawamura M., Hall P. F. and Shively J. E.: Identification of des-(Gly-Ile)-endozepine as an effector of corticotropin-dependent adrenal steroidogenesis: stimulation of cholesterol delivery is mediated by the peripheral benzodiazepine receptor. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 4897–4901.
9. Shoyab M., Gentry L. E., Marquardt H. and Todaro G.: Isolation and characterization of a putative endogenous benzodiazepinoid (Endozepine) from bovine and human brain. *J. Biol. Chem.* **261** (1986) 11,968–11,973.
10. Knudsen J., Hojrup P., Hansen H. O., Hansen H. F. and Roepstorff P.: Acyl-CoA-binding protein in the rat. *Biochem. J.* **262** (1989) 513–519.
11. Chen Z., Agerbeth B., Gell K., Andersson M., Mutt V., Ostenson C-G., Efendic S., Barros-Soderling J., Persson B. and Jornvall H.: Isolation and characterization of porcine diazepam binding inhibitor, a polypeptide not only of cerebral occurrence but also common in intestinal tissues and with effects on regulation of insulin release. *Eur. J. Biochem.* **174** (1988) 239–245.
12. Bovolin P., Schlichting J., Miyata J., Ferrarese C., Guidotti A. and Alho H.: Distribution and characterization of diazepam binding inhibitor (DBI) in peripheral tissues of rat. *Regul. Pept.* **29** (1990) 267–281.
13. Brown A. S., Hall P. F., Shoyab M. and Papadopoulos V.: Endozepine/diazepam binding inhibitor in adrenocortical and Leydig cell lines: absence of hormonal regulation. *Molec. Cell. Endocr.* **83** (1992) 1–9.
14. Garnier M., Boujrad N., Oke B. O., Brown A. S., Riond J., Ferrara P., Shoyab M., Suarez-Quian C. A. and Papadopoulos V.: Diazepam binding inhibitor is a paracrine/autocrine regulator of Leydig cell proliferation and steroidogenesis. Action via peripheral-type benzodiazepine receptor and independent mechanisms. *Endocrinology* **132** (1993) 444–458.
15. Costa E. and Guidotti A.: Diazepam binding inhibitor (DBI): a peptide with multiple biological actions. *Life Sci.* **49** (1991) 325–344.
16. Papadopoulos V., Berkovich A., Krueger K. E., Costa E., Guidotti A.: Diazepam Binding Inhibitor (DBI) and its processing products stimulate mitochondrial steroid biosynthesis via an interaction with mitochondrial benzodiazepine receptors. *Endocrinology* **129** (1991) 1481–1488.
17. Yanagibashi K., Ohno Y., Nakamichi N., Matsui T., Hayashida K., Takamura M., Yamada K., Tou S. and Kawamura M.: Peripheral-type benzodiazepine receptors are involved in the regulation of cholesterol side chain cleavage in adrenocortical mitochondria. *J. Biochem.* **106** (1989) 1026–1029.
18. Brown A. S. and Hall P. F.: Stimulation by endozepine of the side-chain cleavage of cholesterol in a reconstituted enzyme system. *Biochem. Biophys. Res. Commun.* **180** (1991) 609–614.
19. Boujrad N., Hudson J. R. Jr and Papadopoulos V.: Mediation of the hormonal stimulation of steroidogenesis by the polypeptide diazepam binding inhibitor. In *Function of Somatic Cells of the Testis* (Edited by A. Bartke). Springer-Verlag, NY (1994) pp. 186–194.
20. Boujrad N., Hudson J. R. and Papadopoulos V.: Inhibition of hormone-stimulated steroidogenesis in cultured Leydig tumor cells by a cholesterol-linked phosphorothioate oligodeoxynucleotide antisense to diazepam binding inhibitor. *Proc. Natn. Acad. Sci. U.S.A.* **90** (1993) 5728–5731.
21. Garnier M., Boujrad N., Ogwuegbu S. O., Hudson J. R. and Papadopoulos V.: The polypeptide diazepam binding inhibitor and a higher affinity peripheral-type benzodiazepine receptor sustain constitutive steroidogenesis in the R2C Leydig tumor cell line. *J. Biol. Chem.* **269** (1994) 22,105–22,112.
22. Gavish M., Katz Y., Bar-Ami S. and Weizman R.: Biochemical, physiological, and pathological aspects of the peripheral benzodiazepine receptor. *J. Neurochem.* **58** (1992) 1589–1601.
23. Papadopoulos V.: Peripheral-type benzodiazepine/diazepam binding inhibitor receptor: biological role in steroidogenic cell function. *Endocr. Rev.* **14** (1993) 222–240.
24. Mukhin A. G., Papadopoulos V., Costa E. and Krueger K. E.: Mitochondrial benzodiazepine receptors regulate steroid biosynthesis. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 9813–9816.
25. Papadopoulos V., Mukhin A. G., Costa E. and Krueger K. E.: The peripheral-type benzodiazepine receptor is functionally linked to Leydig cell steroidogenesis. *J. Biol. Chem.* **265** (1990) 3772–3779.
26. Krueger K. E. and Papadopoulos V.: Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells. *J. Biol. Chem.* **265** (1990) 15,015–15,022.
27. Papadopoulos V., Guarneri P., Krueger K. E., Guidotti A. and Costa E.: Pregnenolone biosynthesis in C6 glioma cell mitochondria: regulation by a diazepam binding inhibitor mitochondrial receptor. *Proc. Natn. Acad. Sci. U.S.A.* **89** (1992) 5113–5117.
28. Ritta M. N. and Calandra R. S.: Testicular interstitial cells as targets for peripheral benzodiazepines. *Neuroendocrinology* **49** (1989) 262–266.
29. Barnea E. R., Fares F. and Gavish M.: Modulatory action of benzodiazepines on human term placental steroidogenesis *in vitro*. *Molec. Cell Endocr.* **64** (1989) 155–159.
30. Amsterdam A. and Suh B. S.: An inducible functional peripheral benzodiazepine receptor in mitochondria of steroidogenic granulosa cells. *Endocrinology* **128** (1991) 503–510.
31. Holloway C. D., Kenyon C. J., Dowie L. J., Corrie J. E. T., Gray C. E. and Fraser R.: Effect of benzodiazepines diazepam and midazolam on corticosteroid biosynthesis in bovine adrenocortical cells *in vitro*; location of site of action. *J. Steroid Biochem.* **33** (1989) 219–225.
32. Shibita H., Kojima I. and Ogata E.: Diazepam inhibits potassium-induced aldosterone secretion in adrenal glomerulosa cells. *Biochem. Biophys. Res. Commun.* **135** (1986) 994–999.
33. Thompson I., Fraser R. and Kenyon C. J.: Inhibition of bovine adrenocortical steroidogenesis by benzodiazepines: a direct effect on microsomal hydroxylation or an inhibition of calcium uptake. *J. Endocr.* **135** (1992) 361–369.
34. Python C. P., Rossier M. F., Vallotton M. B. and Caponi A. M.: Peripheral-type benzodiazepines inhibit calcium channels and aldosterone production in adrenal glomerulosa. *Endocrinology* **132** (1993) 1489–1496.
35. Thompson I., Fraser R. and Kenyon C. J.: Regulation of adrenocortical steroidogenesis by benzodiazepines. *J. Steroid Biochem. Molec. Biol.* **53** (1994) in press.
36. Papadopoulos V., Nowzari F. B. and Krueger K. E.: Hormone-stimulated steroidogenesis is coupled to mitochondrial benzodiazepine receptors. *J. Biol. Chem.* **266** (1991) 3682–3687.
37. Boujrad N., Gaillard J-L., Garnier M. and Papadopoulos V.: Acute action of choriogonadotropin in Leydig tumor cells. Induction of a higher affinity benzodiazepine receptor related to steroid biosynthesis. *Endocrinology* **135** (1994) 1576–1583.
38. Whalin M. E., Boujrad N., Papadopoulos V. and Krueger K. E.:

Studies on the phosphorylation of the 18 KDa mitochondrial benzodiazepine receptor protein. *J. Rec. Res.* **14** (1994) 217–228.
39. Sprengel R., Werner P., Seeburg P. H., Mukhin A. G., Santi M. R., Grayson D. R., Guidotti A. and Krueger K. E.: Molecular cloning and expression of cDNA encoding a peripheral-type benzodiazepine receptor. *J. Biol. Chem.* **264** (1989) 20,415–20,421.
40. Garnier M., Dimchev A. B., Boujrad N., Price M. J., Musto N. A. and Papadopoulos V.: *In vitro* reconstitution of a functional peripheral-type benzodiazepine receptor from mouse Leydig tumor cells. *Molec. Pharmac.* **45** (1994) 201–211.
41. Riond J., Mattei M. G., Kaghad M., Dumont X., Guillemot J. C., Le Fur G., Caput D. and Ferrara P.: Molecular cloning and chromosomal localization of a human peripheral-type benzodiazepine receptor. *Eur. J. Biochem.* **195** (1991) 305–311.
42. Chang Y. J., McCabe R. T., Rennert H., Budarf M. L., Sayegh R., Emanuel B. S., Skolnick P. and Srauss J. F.: The human "peripheral-type" benzodiazepine receptor: regional mapping of the gene and characterization of the receptor expressed from cDNA. *DNA Cell Biol.* **11** (1992) 471–480.
43. McEnery M. W., Snowman A. M., Trifiletti R. R. and Snyder S. H.: Isolation of the mitochondrial benzodiazepine receptor: association with the voltage-dependent anion channel and the adenine nucleotide carrier. *Proc. Natn. Acad. Sci. U.S.A.* **89** (1992) 3170–3174.
44. Papadopoulos V., Boujrad N., Ikonomovic M. D., Ferrara P. and Vidic B.: Topography of the Leydig cell mitochondrial peripheral-type benzodiazepine receptor. *Molec. Cell. Endocr.* **104** (1994) R5–R9.
45. Mannella C. A., Forte M. and Colombini M.: Toward the molecular structure of the mitochondrial channel, VDAC. *J. Bioenerg. Biomembr.* **24** (1992) 7–19.
46. Bernassau J. M., Reversat J. L., Ferrara P., Caput D. and Lefur G.: A 3D model of the peripheral benzodiazepine receptor and its implication in intra mitochondrial cholesterol transport. *J. Molec. Graphics* **11** (1993) 236–245.
47. Rein H. and Ristau O.: The importance of the high-spin/low-spin equilibrium existing in cytochrome *P*450 for the enzymatic mechanisms. *Pharmazie* **33** (1978) 325–328.
48. Hall P. F.: Cytochrome *P*450 and the regulation of steroid synthesis. *Steroids* **48** (1986) 131–196.
49. Schenkman J. B., Remmer H. and Eastbrook R. W.: Spectral studies of drug interaction with hepatic microsomal cytochrome. *Molec. Pharmac.* **3** (1967) 113–123.
50. Jefcoate C. R.: Cytochrome *P*450 of adrenal mitochondria: steroid binding sites on two distinguishable forms of rat adrenal mitochondrial cytochrome *P*450scc. *J. Biol. Chem.* **252** (1977) 8788–8797.
51. Brown A. S., Leydman M. and Papadopoulos V.: Identification of an ACTH-dependent, diazepam binding inhibitor related, 30 kD protein in bovine adrenocortical inner mitochondrial membranes. *Abstracts of IX International Congress on Hormonal Steroids* (1994) A176.
52. Schultz R., Pelto-Huikko M. and Alho H.: Expression of diazepam binding inhibitor-like immunoreactivity in rat testis is dependent on pituitary hormones. *Endocrinology* **130** (1992) 3200–3206.
53. Rasmussen J. T., Faergeman N. J., Kristiansen K. and Knudsen J.: Acyl-CoA-binding protein can mediate intermembrane acyl-CoA transport and donate acyl-CoA for β-oxidation and glycerolipid syntheiss. *Biochem. J.* **299** (1994) 165–170.
54. Pfanner N., Glick B. S., Arden S. R. and Rothman J. E.: Fatty acylation promotes fusion of transport vesicles with Golgi cisternae. *J. Cell Biol.* **110** (1990) 955–961.
55. Pedersen R. C. and Brownie A. C.: Steroidogenesis activator polypeptide isolated from a rat Leydig cell tumor. *Science* **236** (1987) 188–190.
56. Xu X., Xu T., Robertson D. G. and Lambeth D. J.: GTP stimulates pregnenolone generation in isolated rat adrenal mitochondria. *J. Biol. Chem.* **264** (1989) 17,674–17,680.
57. Pon L. A. and Orme-Johnson N. R.: Acute stimulation of steroidogenesis in corpus luteum and adrenal cortex by peptide hormones. *J. Biol. Chem.* **261** (1986) 6594–6599.
58. Stocco D. M. and Kilgore M. W.: Induction of mitochondrial proteins in MA-10 Leydig tumour cells with human choriogonadotropin. *Biochem. J.* **249** (1988) 95–103.