The Peripheral-type Benzodiazepine Receptor Is Functionally Linked to Leydig Cell Steroidogenesis*

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Testicular mitochondria were previously shown to contain an abundance of peripheral-type benzodiazepine recognition site(s)/receptor(s) (PBR). We have previously purified, cloned, and expressed an M, 18,000 PBR protein (Antkiewicz-Michaluk, Mukhin, A. G., Guidotti, A., and Krueger, K. E. (1988) J. Biol. Chem. 263, 17317-17321; (Sprengel, R., Werner, P., Seeburg, P. H., Mukhin, A. G., Santi, M. R., Grayson, D. R., Guidotti, A., and Krueger, K. E. (1989) J. Biol. Chem. 264, 20415-20421); and in this report, we present evidence that PBR are functionally linked to Leydig cell steroid biosynthesis. A spectrum of nine different ligands covering a range of over 4 orders of magnitude in their affinities for PBR were tested for their potencies to modulate steroidogenesis in the MA-10 mouse Leydig tumor cell line. The Kᵢ for inhibition of the binding of [3H]1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide to the receptor was 54 nM and was not additive to that of epidermal growth factor, another potent ligand for the same receptor, but was additive to dibutyryl cyclic AMP. The EC₅₀ for steroidogenesis for this series of compounds showed a correlation coefficient of r = 0.95. The most potent ligands stimulated steroid production by ~4-fold in these cells. This stimulation was not inhibited by cycloheximide, unlike human chorionic gonadotropin or cyclic AMP-activated steroidogenesis. The action of PBR ligands was not additive to stimulation by human chorionic gonadotropin or cyclic AMP, but was additive to that of epidermal growth factor, another regulator of MA-10 Leydig cell steroidogenesis. Moreover, PBR ligands stimulated, in a dose-dependent manner, pregnenolone biosynthesis by isolated mitochondria when supplied with exogenous cholesterol. This effect was not observed with mitoplasts (mitochondria devoid of the outer membrane). Cytochrome P-450 side chain cleavage activity, as measured by metabolism of (22R)-hydroxycholesterol, was not affected by PBR ligands in intact cells. Similar results were also obtained with purified rat Leydig cells. In conclusion, PBR are implicated in the acute stimulation of Leydig cell steroidogenesis possibly by mediating the entry, distribution, and/or availability of cholesterol within mitochondria.

Benzodiazepines are one of the most widely used classes of drugs in therapy due to their anxiolytic and anticonvulsant properties. It is now well established that besides their interaction with specific recognition sites located in the brain, benzodiazepines bind to membranes prepared from various tissues (1-4) containing the so-called peripheral-type benzodiazepine recognition site(s)/receptor(s) (PBR).¹ Tissue distribution of PBR showed their abundance in the adrenal gland and testis (3, 5, 6). Studies on the intracellular localization of PBR demonstrated that they are predominantly localized on the mitochondria and more precisely on the outer mitochondrial membrane (5-7).

We have recently purified, characterized, and cloned a protein comprising this receptor from rat adrenal gland mitochondria (8, 9). This protein has an M, of about 18,000 and does not show significant sequence similarity to any other currently sequenced protein. Northern analysis has shown the presence of one RNA species of ~850 nucleotides exhibiting relative abundances qualitatively comparable with the densities of PBR in different tissues (9). Furthermore, expression of the corresponding cDNA in eukaryotic cells results in the expression of binding sites for PBR ligands (9).

Despite the accomplishments in elucidating the characteristics of this protein, the physiological role of this class of receptors has remained unknown, although they have been implicated in a number of cellular phenomena such as melanogenesis (10), hemoglobin synthesis (11), inhibition of cell proliferation (12), monocyte chemotaxis (13), protooncogene expression (14), and muscle contraction (15).

The possibility that PBR may play a role in the endocrine regulation of the adrenal gland and testis has first been raised by Anholt et al. (4), who showed that hypophysectomy induces a significant decrease in PBR density in both the adrenal gland and testis. Furthermore, it has been shown that different steroids are able to control the number of PBR in the rat testis (16) and that long-term administration of diazepam increases plasma testosterone levels in men (17), however, it does not have significant effects in rats (18). More direct evidence for a role of benzodiazepines in testicular function has been obtained by in vitro studies on decapsulated testes and interstitial cell suspensions where diazepam and Ro5 4864 have been shown to stimulate androgen production (18-20). But those studies did not demonstrate a direct action of the above-mentioned drugs on Leydig cells, the androgen-producing cell of the testis, or how these drugs act to stimulate

¹ The abbreviations used are: PBR, peripheral-type benzodiazepine recognition site(s)/receptor(s); HCG, human chorionic gonadotropin; EGF, epidermal growth factor; Bt2cAMP, dibutyryl adenosine 3':5'-monophosphate; DBI, diazepam-binding inhibitor; P-450₄₅₀, C₂₅-cholesterol side chain cleavage cytochrome P-450; Ro5-4864, 4'-chloro-diazepam; PK 11195, 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide; PK 1406(7), (-/-)-NJ-diethyl-2-methyl-3-[4-(2-phenyl)quinolinyllpropranamide; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
androgen production. The use of decapsulated testes also 
limits the validity of these results, taking into account the 
regulation of Leydig cell steroidogenesis by paracrine factors 
of Sertoli cell origin (21, 22).

It is important to note that recently it has been reported 
that the endogenous DBI polypeptide, present in most central 
and peripheral tissues, is also expressed in testes (23, 24), and 
its presence has been primarily localized in Leydig cells. DBI 
displaces PBR ligands from rat adrenal gland cortex (25, 26), 
which raises the possibility that DBI may interact physiologi-
ically with PBR and elicit an as yet undefined biological 
activity. Recently, another laboratory (27, 28) has reported 
the purification of an 8.2-kDa polypeptide from bovine adre-
nal fasciculata which exhibited the ability to stimulate preg-
renolone synthesis in mitochondrial preparations of adrenal 
glands. This polypeptide was subsequently sequenced and 
determined to be DBI (29). These findings support the pos-
sibility that PBR and DBI may both cooperate in the process 
of steroid biosynthesis.

The biosynthesis of steroid hormones begins with the trans-
port of the substrate cholesterol from extramitochondrial 
stores to the first enzyme in the pathway, cytochrome P-
450scc, located on the inner mitochondrial membrane. This is 
the rate-limiting step of steroidogenesis and the main site for 
regulation by physiological stimuli during acute stimulation 
(30, 31). The localization of PBR on the mitochondrial com-
partment, the above-mentioned preliminary studies, and the 
finding that DBI is localized in Leydig cells raised the possi-
bility that PBR may participate in the regulation of testicular 
steroidogenesis and prompted us to examine whether PBR 
ligands could alter Leydig cell function. For this purpose, two 
cell models were used: the mouse Leydig cell line MA-10 and 
and purified rat Leydig cells.

**EXPERIMENTAL PROCEDURES**

**Cells**—The MA-10 cell line originally cloned from the solid MA450P 
mouse Leydig cell tumor (32) used in these experiments was gener-
ously given by Dr. Mario Ascoli (The Population Council, Rockefeller 
University, New York). Stock cultures were grown in modified Way-
ham's M199/1 medium containing 5% FBS, 0.2 mM HEPES, 1.2 g/liter 
NaHCO3, and 15% horse serum, pH 7.4, as described by Ascoli (32). 
Before use, the MA-10 Leydig cells grown in 12 × 22-mm wells were 
washed three times, at 30-min intervals, with 1 ml of serum-free 
media to eliminate serum components that may interfere with the 
assays and were incubated for the indicated periods of time in the 
presence of the indicated substances. Testicular interstitial cells were 
prepared by collagenase dissociation (33) of testes obtained from 
adult Sprague-Dawley rats (300 g). This preparation contained 20-
30% 3β-hydroxysteroid dehydrogenase-positive cells (Leydig cells). 
Leydig cells were further purified using discontinuous Percoll gradient 
centrifugation as previously described (33). The preparations obtained 
contained 75–85% Leydig cells as shown by histochemical staining 
for 3β-hydroxysteroid dehydrogenase (33).

**H3PK 11195 Binding Assays and Photolabeling of PBR**—Cells were 
scraped from 75-cm2 culture flasks into 5 ml of Kreb's buffer (154 mM 
NaCl, 60 mM HCO3, 5 mM CaCl2, 1.0 mM KH2PO4, 1.5 mM 
MgSO4, 20 mM NaPO4, pH 7.4), dispersed by trituration, and cen-
trifuged at 12,000 × g for 5 min. The cell pellets were resuspended 
in buffer, and larger cell aggregates were allowed to settle to the bottom 
of the tube before the cell suspension was removed for experimenta-
tion.

**H3PK 11195 binding studies on 5 μg of protein from the cell 
suspensions were performed in 250–400 μl of Kreb's buffer at 37 °C, 
especially in conditions under which the effects of PBR ligands on 
steroidogenesis were studied. Non specific binding was 
determined in the presence of 10 μM PK 11195. After 30 min, the 
assays were stopped by filtration through Whatman GF/C filters and 
and washed with 20 ml of 25 mM Tris-HCl, pH 7.4, containing 1 μM PK 
11195. Radioactivity trapped on the filters was determined by liquid 
cintillation counting. Total binding accounted for <10% of the 
radioligand introduced, whereas specific binding was >85% of 
the total binding at all radioligand concentrations used.

**Photolabeling of MA-10 cell mitochondria was performed by sus-
pending 2 mg of membrane protein in 2 ml of 25 mM Tris-HCl, pH 
7.4, 0.32 mM sucrose buffer which contained 9 nM [3H]PK 14105. The 
membranes were preincubated at 4 °C for 30 min before being placed 
in a rectangular dish (75 × 52 × 5 mm), continuously rocked for 60 
min at 4 °C, and simultaneously irradiated from a distance of 2 cm 
using an UVOL-58 ultraviolet light (UVF, Inc., Sau, Gabriel, CA) 
with maximum emission at 366 nm. Following irradiation, 10 μl of 2 
mM PK 11195 was added to the membrane suspensions. The samples 
were reincubated for another 30 min, diluted into 10 ml of Tris/sucrose 
buffer, and centrifuged at 20,000 × g for 10 min; and the pellets were 
 washed once more in 10 ml of buffer.

**Steroid Biosynthesis**—These experiments were performed with the 
MA-10 Leydig cells plated in 12 × 22-mm wells and incubated for the 
times shown in the presence of the indicated substances at a final 
volume of 1 ml of serum-free media at 37 °C. Where purified rat 
Leydig cells were used, 50,000 cells/500 μl of serum-free media were 
incubated in bovine serum culture tubes at 32 °C. At the end of the 
incubation period, the cell media were saved, centrifuged at 1,500 × 
g for 10 min, and stored at −20 °C until used. Cells were dissolved 
with 0.1 N NaOH for protein measurement.

**Progesterone and 20α-hydroxyprogesterone production for the 
MA-10 cells and testosterone accumulation for the rat Leydig cells 
were measured by means of radioimmunoassay. Antibody to proges-
terone was obtained from Endocrine Sciences (Tarzana, CA), and the 
assay was performed as described by the manufacturer. Antibody to 
20α-hydroxyprogesterone was a gift from Dr. G. Nieswender (Colo-
rado State University), and the assay was performed as described 
(34). Anti-testosterone antibody was obtained from ICN Co. (Lisle, 
IL), and the assay was performed as described by the manufacturer. 
Analysis of the radioimmunoassay data was performed using the 
IBM-PC RIA Data Reduction program (version 4.1) obtained from 
Jaffe and Associates (Silver Spring, MD).

**Measurement of Cholesterol Transport in Mitochondria—MA-10 
Leydig cells were washed with serum-free media as described above 
followed by washing in phosphate-buffered saline. Mitochondria were 
then prepared as previously described (35, 36). The effect of different 
compounds on cholesterol transport and/or on P-450scc activity 
was determined as previously described (28, 36) by incubating mitochon-
drial with the ligands in the presence of exogenous cholesterol (100 
μM) and triostane (0.1 μM), an inhibitor of pregnenolone metabolism 
(37). The production of pregnenolone was measured after a 10-min 
icubation with a specific radioimmunoassay (38) using an antibody 
given by Dr. F. P. G. Rommens (Erasmus University, Rotterdam, 
Netherlands). In another series of experiments, MA-10 Leydig cell 
mitochondria were prepared by a method of Borsani and Cavet (39) 
(see also Ref. 36) and incubated under the same conditions described 
above for mitochondria. The purity of mitochondria was 
examined by measuring cytochrome-c oxidase and Amaryl-insen-
sitive NADPH-cytochrome-c reductase as markers for inner and outer 
mitochondrial membranes, respectively (36). Values for these en-
yzymes in mitoplasts were 4.2 ± 0.5 mol/min/mg of protein and 
38 ± 
23 nmol/min/mg of protein corresponding to a 3-fold increase and 
a 10-fold decrease, respectively, of the specific activity for each 
enzyme compared to whole mitochondria.

**Protein Measurement—Protein was measured by the method of 
Bradford (40) using bovine serum albumin as a standard.

**Photolabeled—Purified human chorionic gonadotropin (hCG), batch 
CR-125 with a biological potency of 11,000 IU/mg) was a gift from 
the National Institutes of Health. Purified mouse epidermal growth 
factor (EGF) was purchased from Sigma. [3H]Progesterone (specific 
activity of 41 Ci/mmol), 20α-[3H]Hydroxyprogesterone (specific 
activity of 45 Ci/mmol), [1,2,6,7-3H]Testosterone (specific 
activity of 75 Ci/mmol), [5-3H]Pregnenolone (specific 
activity of 95 Ci/mmol), and [3H]Pregnanetriol (specific 
activity of 22.6 Ci/mmol), and N-[3H]-[111]PK 11195 were obtained from Du 
Pont-New England Nuclear. [3H]PK 14105 was purchased from Re-
search Products International Corp. PK 11106, PK 14067, and PK 
14068 were the gift of Dr. C. Gueremy (Pharmuka Laboratoire 
Antonin, Paris, France). Diazepam, clonazepam, flumazenil, and Ro5-4864 were a gift from Hoffman-La Roche. Aminoglutethimide was 
provided by Ciba Geigy, and trilostane was from Sterling-Winthrop 
(New York, NY).

Percoll was purchased from Pharmacia LKB Biotechnology Inc. All
cell culture supplies were purchased from GIBCO, and cell culture plasticware was from Corning. All other chemicals were of analytical quality and were obtained from commercial sources.

RESULTS

Characterization of PBR in MA-10 Cells—Kinetics of [3H]PK 11195 binding at 37 °C to MA-10 cells revealed that specific binding of this ligand was rapid, reaching steady-state conditions after 10 min (data not shown). Furthermore, if 10 μM PK 11195 was subsequently added, specific radioligand binding was completely displaced within 15–20 min. Scatchard analysis demonstrated a single class of binding sites with a dissociation constant of 1.8 nM at a density of 56 pmol/mg of protein (Fig. 1). Photolabeling of MA-10 cell mitochondrial fractions with [3H]PK 14105 verifies that these cells contain the 18-kDa protein which constitutes the binding domains for PBR ligands (9).

Specificity of these binding sites was determined using nine different ligands from several classes of organic compounds known to interact with PBR. These ligands exhibited a rank order potency to compete against [3H]PK 11195 binding which is consonant with their relative potencies reported by other groups (Fig. 2). The most notable observations are that the isoquinoline carboxamide PK 1195 was the most potent compound of this series. The quinoline propramamide PK 14067 was about 200-fold more potent than its enantiomer, PK 14068 (41). Of the benzodiazepines, Ro5-4864 was the most potent derivative, whereas clonazepam and flumazenil, an antagonist of central benzodiazepine actions, exhibited low affinities for PBR.

Effect of PBR Ligands on MA-10 Leydig Cell Steroid Biosynthesis—To investigate whether the different ligands affected Leydig cell steroidogenesis, increasing concentrations of these compounds were incubated for 4 h with MA-10 cells, and their effects on progesterone production were measured (Fig. 3). The three most potent compounds of this series were very efficacious at stimulating progesterone synthesis, whereas the least potent compounds either achieved a lower level of maximal stimulation or did not have an effect on steroid production. PK 1195 and PK 14067 were the most potent and produced a 3–4-fold stimulation of progesterone production. Among the benzodiazepines used, Ro5-4864 was the most potent, giving a 3.5-fold increase in steroidogenesis, whereas diazepam attained only a 2-fold maximal stimulation.

When comparing the EC50 values of the stimulatory effect of the different PBR ligands with the inhibitory constants with which these ligands compete for [3H]PK 11195 binding to the MA-10 cells (Fig. 4), an excellent correlation was observed (r = 0.95). This provides strong evidence that the effects of these drugs on steroidogenesis are consequent to their binding to PBR. PK 11195 (10−8 M) and Ro5-4864 (10−5 M) also stimulated 20α-hydroxyprogesterone production by MA-10 Leydig cells to similar extents as observed with progesterone (data not shown), indicating that their actions are not unique to progesterone and that there is an increase of all steroidogenic products of these cells. This implies an action at the level of the initial substrate (cholesterol) and its processing. It is important to note at this point that all compounds used in these studies did not cross-react with the antibodies used in the different radioimmunoassays.

Effect of PBR Ligands on hCG- and EGF-stimulated Ste-
Leydig Cell Steroidogenesis Mediated by PBR

FIG. 4. Correlation between binding affinities and stimulatory potencies of PBR ligands. The $K_i$ estimated for each compound from the analysis of Fig. 2 is compared to the concentration of ligand required to exhibit one-half of the respective maximal stimulation as was determined in Fig. 3. Point 1, PK 11195; point 2, PK 14067; point 3, Ro5-4864; point 4, valproate; point 5, PK 14068; point 6, diazepam; point 7, flunitrazepam.

FIG. 5. Effects of PBR ligands on hCG- and EGF-stimulated steroidogenesis. MA-10 Leydig cells were incubated for 4 h in the presence of $10^{-6}$ M PK 11195 (A) or $10^{-5}$ M Ro5-4864 (B) with increasing concentrations of hCG (A, ●) or EGF (B, ●). Cell media were collected, and progesterone accumulation was measured by radioimmunoassay. Values are means ± S.D. of two independent experiments ($n = 4$). Note the different units used on the abscissas for both panels.

FIG. 6. Time course on effects of PBR ligands, hCG, and EGF on MA-10 steroid biosynthesis. MA-10 Leydig cells were incubated for the times shown in the absence (○) or presence of $10^{-6}$ M PK 11195 (●), $10^{-5}$ M Ro5-4864 (□), $10$ ng/ml EGF (●), or $50$ ng/ml hCG (▲). At the end of the incubation period, media were collected, and progesterone was measured by radioimmunoassay. Values are means ± S.D. from two independent experiments ($n = 4$) plotted on two separate scales. In parallel experiments, incubation of the cells with $1$ mM Bt+AMP gave results which were equivalent to those with hCG.

Since hCG is an unusually potent activator of steroidogenesis in MA-10 Leydig cells in comparison with normal Leydig cells, we also examined the effect of PBR ligands on the responses of Leydig cells to EGF, another modulator of their function. In Fig. 5B, a dose-response curve of EGF on Leydig cell progesterone production is shown. Maximal stimulation of steroidogenesis (6-fold) was obtained with $10$ ng/ml EGF. PK 11195 and Ro5-4864 increased Leydig cell steroid output in the presence of all concentrations of EGF used, indicating that EGF and PBR ligands stimulate steroidogenesis in MA-10 Leydig cells by two different mechanisms.

Time course studies on the effect of the steroidogenic active PBR ligands on Leydig cell progesterone production were undertaken and compared with the results obtained from time course studies using hCG and EGF (Fig. 6). As early as 10 min following exposure to the cells, PK 11195 and Ro5-4864 stimulated steroid biosynthesis above the basal rate of synthesis; and after 40 min progesterone synthesis had reached its completion. In contrast, the stimulation observed with EGF exhibited a slower response, and maximal steroid accumulation was achieved after 3 h. It is important to point out that hCG-stimulated progesterone production was also observed within 10 min, as was seen with the PBR ligands, but it reached a plateau after 4 h (Fig. 6). These findings further support the implication that PBR ligands act through a mechanism different from that employed by EGF but which...
may be included in the multiple cellular events affected by hCG.

Studies on Mechanism of Action of PBR Ligands on Leydig Cell Steroidogenesis—In search for the site of action of PBR ligands on steroid biosynthesis, we first examined the possibility that PBR ligands may affect protein synthesis, which in turn will affect steroidogenesis. In the presence of the protein synthesis inhibitor cycloheximide (0.2 mM), hCG or Bt-cAMP-stimulated steroidogenesis is almost completely inhibited, although a slight but significant stimulation remains (Table I). In contrast, progesterone production stimulated by 10−6 M PK 11195 or 10−5 M Ro5-4864 was not inhibited by cycloheximide. At submaximal concentrations of hCG (0.5 ng/ml), steroid production was still greater than that observed with the PBR ligands; cycloheximide, however, completely inhibited steroid production at these hCG concentrations (Table I). These findings demonstrate that cycloheximide does not inhibit the stimulation by PBR ligands, unlike the stimulation observed with hCG.

Table I also shows that stimulation of steroid biosynthesis by hCG, Bt-cAMP, or PBR ligands is inhibited by the P-450, inhibitor aminoglutethimide (42, 43). Furthermore, progesterone production under conditions of unlimited substrate, as in the presence of exogenous pregnenolone, was not affected by hCG, Bt-cAMP, or PBR ligands (data not shown), indicating that their acute regulatory effects on steroidogenesis are prior to pregnenolone metabolism.

At this point, we also examined the possibility that ligand binding to PBR may affect cholesterol metabolism to pregnenolone by directly acting on the mitochondrial P-450, enzyme. The metabolism of (22R)-hydroxycholesterol to progesterone by MA-10 cells was used as an index for P-450, activity (44). Ro5-4864 (10−5 M) did not affect progesterone production in the presence of 0.25 μM (22R)-hydroxycholesterol (244.67 ± 42.52 versus 239.67 ± 30.33 ng of progesterone/mg of protein/4 h; means ± S.D., n = 3) or in the presence of 100 μM (22R)-hydroxycholesterol (maximal concentration) (5047.00 ± 238.04 versus 4977.33 ± 213.30 ng of progesterone/mg of protein/4 h; means ± S.D., n = 3). Similar results were observed using other (22R)-hydroxycholesterol concentrations (data not shown), showing that the mechanism of stimulation by PBR is not by direct activation of P-450,.

These findings and the fact that PBR are essentially located on the outer mitochondrial membrane prompted us to examine whether this receptor plays a role in cholesterol transport, the rate-limiting step of steroidogenesis.

To investigate the effect of PBR in cholesterol transport in vitro, PBR ligands were added to Leydig cell mitochondria incubated in the presence of exogenous cholesterol and trilostane, an inhibitor of 3β-hydroxysteroid dehydrogenase. Fig. 7A shows the results obtained from these experiments. PK 11195 and Ro5-4864, in the same concentration ranges that increased intact MA-10 cell steroidogenesis, stimulated pregnenolone production in Leydig cell mitochondria, whereas 10−5 M clonazepam was without any effect. To ensure that these effects were mediated through the outer mitochondrial membrane (the site where PBR are localized), similar experiments were performed with mitoplasts (mitochondrial preparations from which the outer mitochondrial membrane was removed). Mitoplasts therefore contain the inner mitochondrial membrane with its constituent P-450, activity. The results obtained (Fig. 7B) showed that PBR ligands did not have an effect on pregnenolone production from mitoplasts, further demonstrating that they do not act directly on the P-450, enzyme. Therefore, from these findings, it can be deduced that PBR are most likely involved with cholesterol uptake from intracellular stores into mitochondria and/or promoting cholesterol availability to P-450,.

Table I shows the results obtained from these experiments. PK 11195 and Ro5-4864 (10−5 M) did not affect steroidogenesis and the substances indicated. Therefore, from these findings, it can be deduced that PBR are most likely involved with cholesterol uptake from intracellular stores into mitochondria and/or promoting cholesterol availability to P-450,.

**Regulation of Steroidogenesis by PBR Ligands in Leydig Cells Purified from Rat Testis**—To exclude the possibility that the involvement of PBR in steroidogenesis may be specific to the MA-10 cell line rather than representative of normal Leydig cells, we also examined the action of PBR ligands on purified rat Leydig cell steroidogenesis. Table II shows that

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**Table I**

**Effects of cycloheximide and aminoglutethimide on MA-10 Leydig cell steroidogenesis**

MA-10 Leydig cells were incubated for 4 h with or without cycloheximide or aminoglutethimide and the substances indicated. Progesterone production was measured by radioimmunoassay in cell media. Results are the means ± S.D. from two experiments (n = 4).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No additions</th>
<th>Cycloheximide (0.2 mM)</th>
<th>Aminoglutethimide (0.76 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nG/mg protein/h</td>
<td>nG/mg protein/h</td>
</tr>
<tr>
<td>Control</td>
<td>13.25 ± 2.50</td>
<td>12.25 ± 2.87</td>
<td>13.50 ± 2.64</td>
</tr>
<tr>
<td>hCG (60 ng/ml)</td>
<td>4823 ± 131</td>
<td>33.25 ± 13.12</td>
<td>14.50 ± 3.69</td>
</tr>
<tr>
<td>Bt-cAMP (1 mM)</td>
<td>4772 ± 245</td>
<td>37.00 ± 10.86</td>
<td>13.75 ± 2.21</td>
</tr>
<tr>
<td>hCG (0.5 ng/ml)</td>
<td>501 ± 10</td>
<td>13.40 ± 2.56</td>
<td>14.10 ± 2.83</td>
</tr>
<tr>
<td>PK 11195 (10−6 M)</td>
<td>49.00 ± 6.27</td>
<td>47.00 ± 7.61</td>
<td>16.00 ± 0.81</td>
</tr>
<tr>
<td>Ro5-4864 (10−5 M)</td>
<td>45.50 ± 4.43</td>
<td>45.50 ± 6.02</td>
<td>15.50 ± 1.73</td>
</tr>
</tbody>
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**Fig. 7.** Effect of PBR ligands on pregnenolone formation by MA-10 Leydig cell mitochondria and mitoplasts. Mitochondria (A) or mitoplasts (B) were incubated with cholesterol (100 μM), trilostane (0.1 μM), and the indicated concentrations of PK 11195 (Δ) or Ro5-4864 (C) or clonazepam. Incubations were performed for 10 min at 37 °C, and pregnenolone was then measured by radioimmunoassay. Note that in A the values for clonazepam are not shown since they are equal to those obtained in the absence of ligands. Results of one representative experiment are shown. Values are means ± S.D. derived from triplicate determinations.
PK 11195 and Ro5-4864 stimulate, by about 2-fold, testosterone production by purified rat Leydig cells, whereas diazepam was less potent, and clonazepam had no effect. Moreover, stimulation of rat Leydig cell steroidogenesis was stereoselective for PBR, as demonstrated using the optical isomers PK 14067 and PK 14068. The hCG-stimulated testosterone production was not affected by PBR ligands (Table II). The site of PBR ligand action in rat Leydig cell steroid biosynthesis was also located, as was performed in MA-10 cells, at the level of cholesterol transport into mitochondria, the rate-limiting step of steroidogenesis (data not shown). These results verify that the effects on steroidogenesis mediated by PBR are also found in normal rat Leydig cell preparations. Hence, the MA-10 cell line has proven to be a suitable model system to examine the role of PBR in Leydig cell function.

**DISCUSSION**

The experiments reported here were designed to investigate the role of PBR in Leydig cell function. It has been previously shown (3, 5–7) that PBR are abundant in the outer mitochondrial membrane and are predominantly localized on the outer mitochondrial membrane. A number of preliminary reports (18–20, 29, 45) show that some PBR ligands can increase steroidogenesis; however, a detailed pharmacological analysis was lacking to establish whether the mediator of this action was PBR. This study establishes that PBR play an important role in steroid biosynthesis and that their subcellular localization is consistent with current knowledge on the mitochondrial compartments participating in steroidogenesis.

In vitro studies using decapsulated testes or interstitial cell suspensions have shown that benzodiazepines (diazepam and Ro5-4864) stimulate androgen production (18–20). In view of the complexity of testicular structure and the role of cell-cell interactions in the testis (21, 22), we decided to examine the participation of PBR in Leydig cell steroidogenesis. For this purpose, two model systems, the mouse tumor MA-10 cell line and Leydig cells purified from rat testes, were used to demonstrate an excellent correlation between the affinities of nine ligands for PBR and their potencies to stimulate steroidogenesis.

MA-10 cells were found to contain a density of PBR which corresponds to ~6 x 10^6 binding sites/cell, representing about 0.1% of the total cell protein based on the M, ~18,000 determined from our earlier PBR purification and cDNA cloning studies (8, 9) and verified here by photolabeling experiments with [1H]PK 14105. This extreme abundance of PBR is comparable with the levels found in adrenal gland mitochondria (6) and is consistent with the high density of these receptors found in apparently all steroidogenic cells (5).

Among the ligands within the series which were tested, PK 11195, PK 14067, and Ro5-4864 exhibited both the highest affinities for PBR and the greatest maximal stimulation of steroid production. Our data reveal only one class of PBR in MA-10 cells, yet the ligands with lower affinities for PBR elicit a lesser maximal stimulation despite the fact that they completely displace [1H]PK 11195 from PBR, with a Hill coefficient of near unity. This implies that the affinity a ligand has for PBR is an important determinant for the magnitude of the steroidogenic response which is achieved. It should also be noted that none of the compounds acted as antagonists, unlike the reports of others (13, 15) examining different functions for PBR. This difference might indicate structural or functional heterogeneity of PBR in different cell systems.

Since hCG produces a very robust stimulation (>300-fold) of steroidogenesis in MA-10 cells (32), a response which is atypical for normal Leydig cells (10-fold stimulation), one might argue that the effects of PBR ligands are very modest and possibly insignificant. This argument was ruled invalid because purified rat Leydig cells also exhibited a significant stimulation of testosterone synthesis in response to PBR ligands. In every respect which we examined, the effects of PBR ligands in purified Leydig cells or their isolated mitochondria were equivalent to the effects observed in MA-10 cells.

For these studies, we used the accumulation of extracellular progesterone as an index of steroid biosynthesis for MA-10 cells (32). The observations that the production of 20a-hydroxyprogesterone, another steroid product of MA-10 cells, was increased at the same level as progesterone and that the metabolism of exogenous pregnenolone and hydroxysterol cholesterol was not altered after addition of ligands indicated that the site of action of PBR ligands was likely to be prior to pregnenolone formation.

Steroid biosynthesis begins with the transport of cholesterol from extramitochondrial stores into mitochondria, where it is metabolized to pregnenolone by P-450, located on the inner mitochondrial membrane (30, 46, 47). This delivery is the rate-limiting step of steroidogenesis and the main site of action of the acute stimulation by gonadotropin (48). The role of PBR was found to be consistent with its proposed outer mitochondrial membrane localization as PBR ligands had no effect in mitoplasts but did apparently increase cholesterol delivery to P-450, in intact mitochondria as evidenced by the stimulation of pregnenolone synthesis. More studies are required to elucidate the precise step of the pathway in which PBR function. Based on the studies presented here, PBR could play a role in mitochondrial uptake of cholesterol into a steroidogenic pool and/or transport of cholesterol from the outer to inner mitochondrial membrane. We excluded the possibility that ligand binding to PBR may result in a direct action on P-450, activity since addition of PBR ligands to Leydig cells did not affect metabolism of (22R)-hydroxycholesterol to progesterone.

When PBR ligands were added together with hCG to MA-10 cells, an additive effect was not seen. This implies that hCG and PBR may act through a common pathway to increase steroid biosynthesis. Since hCG is known to activate multiple cellular events in triggering steroidogenesis, one site of action might include the participation of PBR in the hormone-mediated response. However, since other systems are also affected in the hCG-mediated response, this would account for the differences in kinetics, magnitude of stimulation, and sensitivity to cycloheximide between hCG and PBR ligands.

It had been demonstrated (31, 49, 50) that cycloheximide...
blocks hormone-stimulated steroid production and that the inhibition occurs at the site of cholesterol transport into mitochondria. The results in Table I show that cycloheximide does not inhibit ligand-stimulated progesterone production by MA-10 Leydig cells, whereas it essentially abolishes hCG-stimulated steroidogenesis, in agreement with previous results (61). This suggests that promotion of cholesterol transport elicited by PBR ligands is not sensitive to cycloheximide, presumably due to a direct activation of PBR. The kinetics of ligand binding and steroidogenic activity we observed support this proposal. These findings might help account for the suggestions of others (52–54) that hCG-stimulated cholesterol transport is not entirely dependent on a protein synthesis-mediated step. In retrospect, it is also possible that the hormone response may include a cycloheximide-sensitive step to recruit PBR for steroid biosynthesis, and receptor occupancy by ligands merely overrides this physiological control.

In contrast to their relationship with the stimulation by hCG, PRR ligands have an additive effect on steroidogenesis with EGF, another modulator of Leydig cell steroidogenesis (55, 56). It has been reported by Ascoli et al. (55) that EGF and submaximal concentrations of hCG stimulate progesterone production by MA-10 Leydig cells using two different pathways. Our results show that PBR ligands and EGF also use different pathways to increase steroid biosynthesis. Moreover, the kinetics of drug action on progesterone production is much more rapid than that of EGF, indicating a more direct action on a regulatory step of steroidogenesis.

While the experiments presented here were in progress, a paper was published by Besman et al. (29) showing that diazepam, a ligand for central and peripheral benzodiazepine recognition sites, stimulates pregnenolone formation by bovine adrenal gland mitochondria at a concentration of ~10 μM. Our studies on Leydig cells with diazepam are in agreement with these findings, except that in our model, the EC50 of the stimulation obtained with diazepam is ~1 μM. Since we obtained similar values with rat and mouse adrenal gland cells (data not shown), this discrepancy with bovine adrenal gland cells is probably due to the known species differences in receptor affinity for benzodiazepines (57).

In search for physiological endogenous ligands for benzo- diazepine receptors, another laboratory at this institution has found that DBI, a polypeptide abundant in steroidogenic tissues, displaces the PBR ligand, androstenedione, from rat adrenal gland mitochondrial membranes (25, 26). In addition, the workers found that DBI is expressed in the testis (29, 34), where it primarily localizes in Leydig cells. We have also measured high levels of immunoreactive DBI in MA-10 Leydig cells (data not shown). Furthermore, there are recent reports (28, 29) that a protein from bovine adrenal fasciculata cells which stimulates steroidogenesis was identified as being DBI. These new developments raise the possibility that DBI may interact physiologically with PBR mediating cholesterol delivery into mitochondria, thereby activating steroidogenesis. The uncovering of these potential components in the steroidogenic pathway may prove to be an important step in elucidating the molecular systems which regulate steroid biosynthesis.

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