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 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 [³H]1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide binding and the EC₅₀ for steroid biosynthesis 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.

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**The abbreviations used are: PBR, peripheral-type benzodiazepine recognition site(s)/receptor(s); HCG, human chorionic gonadotropin; EGF, epidermal growth factor; B₄₅CAMP, dibutylryl adenosine 3'5'-monophosphate; DBI, diazepam-binding inhibitor; P-450, cytochrome P-450; hCG, human chorionic gonadotropin; DBI, diazepam-binding inhibitor; P-450, cytochrome P-450; hCG, human chorionic gonadotropin.
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.5 DBI displaces PBR ligands from rat adrenal gland cortex (25, 26), which raises the possibility that DBI may interact physiologically 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 adrenal fasciculata which exhibited the ability to stimulate pregnenolone synthesis in mitochondrial preparations of adrenal glands. This polypeptide was subsequently sequenced and determined to be DBI (29). These findings support the possibility that PBR and DBI may both cooperate in the process of steroid biosynthesis.

The biosynthesis of steroid hormones begins with the transport 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 compartment, the above-mentioned preliminary studies, and the finding that DBI is localized in Leydig cells raised the possibility 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 purified rat Leydig cells.

**Experimental Procedures**

**Cells**—The MA-10 cell line originally cloned from the solid M572/P mouse Leydig cell tumor (32) used in these experiments was generously given by Dr. Mario Ascoli (The Population Council, Rockefeller University, New York). Stock cultures were grown in modified Waymouth's MB 752/1 medium containing 3.5% fetal bovine 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-hydroxy steroid 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 (35).

**[3H]PK 11195 Binding Assays and Photolabeling of PBR**—Cells were scraped from 75-cm2 culture flasks into 5 ml of Kreb's buffer (124 mM NaCl, 60 mM KCl, 3 mM CaCl2, 1.2 mM KH2PO4, 1.5 mM MgSO4, 20 mM NaHCO3, pH 7.4), dispersed by trituration, and centrifuged at 1200 × 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 retrieved for experimenta-

**[3H]PK 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, essentially under conditions unique with the effects of PBR ligands on steroidogenesis were studied. Nonspecific 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 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 scintillation 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 μg of membrane protein in 2 ml of 25 mM Tris-HCl, pH 7.4, 0.32 M 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., Saul 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 incubated 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/50 μl of serum-free media were incubated in borosilicate 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.

**Growth and 20α-hydroxyprogesterone production for the MA-10 cells and testosterone accumulation for the rat Leydig cells** were measured by means of radioimmunoassay. Antbody to progesterone 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 (Colorado 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).

**Photolabeling of Mitochondria in Culture**—MA-10 Leydig cells were washed with serum-free media as described above following 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 mitochondria with the ligands in the presence of exogenous cholesterol (100 μM) and trilostane (0.1 μM), an inhibitor of pregnenolone metabolism (37). The production of pregnenolone was measured after a 10-min incubation with a specific radioimmunoassay (38) using an antibody given by Dr. F. F. G. Rommerts (Erasmus University, Rotterdam, Netherlands). In another series of experiments, MA-10 Leydig cell mitochondria were prepared by the method of Wieland and Geisawalt (Ref. 39; see also Ref. 35) and incubated under the same conditions described above for mitochondria. The purity of mitoplasts was examined by measuring cytochrome-c oxidase and Amyal-l-insensitive NADH:cytochrome-c reductase as markers for inner and outer mitochondrial membranes, respectively (38). Values for these enzymes in mitoplasts were 4.2 ± 0.5 mol/min/mg of protein and 38 ± 23 mmol/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.

**Purified tubular mitochorndial ghosts (MG)**—batch CR-125 with a biological potency of 11,000 IU/mg was a gift from the National Institutes of Health. Purified mouse epididymal epidermal growth factor (RGEF) was purchased from Sigma (19,6,7-3H)Progestergone (specific activity of 94 Ci/mmol), 20α-[1,2-3H]Hydroxyprogesterone (specific activity of 45 Ci/mmol), [1,2,6,7-3H]Testosterone (specific activity of 95.9 Ci/mmol), [3H]Pregnenolone (specific activity of 9.5 Ci/mmol), [3H]Squalene (specific activity of 22.6 Ci/mmol), and N-[methyl]-3H]PK 11195 were obtained from Du Pont-New England Nuclear. [3H]PK 14105 was purchased from Research Products International Corp. PK 11195, PK 14067, and PK 11195 were purchased by the method of Bradford (40) using bovine serum albumin as a standard.

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 \(^{[3]H}\)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 nM 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 \(^{[3]H}\)PK 14105 verified 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 \(^{[3]H}\)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 11195 was the most potent compound of this series. The quinoline propranamide 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 Steroidogenesis—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 11195 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. The other compounds used significantly stimulated steroid production (1.5-fold) only at concentrations higher than 10^{-6} M, whereas clonazepam and flumazenil were completely inactive at concentrations above 10^{-5} M.

When comparing the EC_{50} values of the stimulatory effect of the different PBR ligands with the inhibitory constants with which these ligands compete for \(^{[3]H}\)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^{-6} 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

-8 -7 -6 -5 Log K, (Binding)

**FIG. 4.** Correlation between binding affinities and stimulatory potencies of PBR ligands. The K, 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, zolpidem; 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 ( dol), 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.

**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 (△) or 10^{-5} M Ro5-4864 (□) 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.

Steroidogenesis—MA-10 Leydig cells respond to hCG in a dose-dependent manner, with a half-maximal stimulation at 3–5 ng/ml (Fig. 5A). Maximal stimulation of progesterone production (30-fold) was obtained with 25 ng/ml hCG. Addition of 10^{-6} M PK 11195 or 10^{-5} M Ro5-4864 to increasing amounts of hCG did not affect the responses obtained by hCG alone (Fig. 5A), indicating that PBR ligands and hCG may act through a common pathway. To further examine whether PBR ligands may modulate the Leydig cell response to this hormone, 0.5 ng/ml hCG was used in the presence and absence of increasing amounts of PK 11195. In two separate experiments, we were unable to show any modification of hCG (0.5 ng/ml)-stimulated progesterone production by MA-10 cells at concentrations of PK 11195 up to 10^{-6} M (206 ± 17.8 ng of progesterone/mg of protein without PK 11195 versus 221 ± 19.7 ng of progesterone/mg of protein with 10^{-6} M PK 11195).

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^{-6} 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 
 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 µ M (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 µ M (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.

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 µ M (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 µ M (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 enzyme. 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.

**Regulation of Steroidogenesis by PBR Ligands in Leydig Cells Purified from Rat Testes**—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...

**Table I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Progesterone (ng/mg protein/4 h)</th>
<th>Cyclheximide (0.2 mM)</th>
<th>Aminoglutethimide (0.76 mM)</th>
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</thead>
<tbody>
<tr>
<td>No additions</td>
<td>13.25 ± 2.50</td>
<td>12.25 ± 2.87</td>
<td>13.50 ± 2.64</td>
</tr>
<tr>
<td>hCG (50 ng/ml)</td>
<td>4852 ± 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>201 ± 16</td>
<td>13.40 ± 2.56</td>
<td>14.10 ± 2.83</td>
</tr>
<tr>
<td>PK 11195 (10^{-5} M)</td>
<td>49.00 ± 0.87</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>
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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 µ M), trilostane (0.1 µ M), and the indicated concentrations of PK 11195 (Δ) or Ro5-4864 (○) 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.
with [3H]PK 14105. This extreme abundance of PBR is mined from our earlier PBR purification and cDNA cloning (6) and is consistent with the high density of these receptors found in apparently all steroidogenic cells (3). Comparable with the levels found in adrenal gland mitochondria (0.1% of the total cell protein based on the ligands for PBR and their potencies to stimulate steroidogenesis), demonstrate an excellent correlation between the affinities of nine 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 [3H]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).

TABLE II
Effects of PBR ligands on basal and hCG-stimulated rat Leydig cell steroidogenesis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Testosterone (ng/10^6 cells/4 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.30 ± 0.20</td>
</tr>
<tr>
<td>PK 11195 (10^-6 M)</td>
<td>2.33 ± 0.21</td>
</tr>
<tr>
<td>PK 14067 (10^-6 M)</td>
<td>2.20 ± 0.30</td>
</tr>
<tr>
<td>PK 14068 (10^-5 M)</td>
<td>1.16 ± 0.15</td>
</tr>
<tr>
<td>Ro5-4864 (10^-6 M)</td>
<td>2.40 ± 0.30</td>
</tr>
<tr>
<td>Diazepam (10^-6 M)</td>
<td>1.90 ± 0.10</td>
</tr>
<tr>
<td>Clonazepam (10^-6 M)</td>
<td>1.16 ± 0.11</td>
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</table>

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 [3H]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 hydroxyl cholesterol was not altered after addition of ligands indicated that the site of action of PBR ligands is 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 (51, 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 observations 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, PBR 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 DBI, a polypeptide abundant in steroidogenic tissues, displaces the PBR ligands from rat 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 benzodiazepine receptors, another laboratory at this institution has found that DBI, a polypeptide abundant in steroidogenic tissues, displaces the PBR ligands from rat adrenal gland mitochondrial membranes (25, 26). In addition, the workers found that DBI is expressed in the testis (29, 24), 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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