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Peripheral-Type Benzodiazepine Receptor-Mediated Action of Steroidogenic Acute Regulatory Protein on Cholesterol Entry into Leydig Cell Mitochondria

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Hormone-induced steroid biosynthesis begins with the transfer of cholesterol from intracellular stores into mitochondria. Steroidogenic acute regulatory protein (StAR) and peripheral-type benzodiazepine receptor (PBR) have been implicated in this rate-determining step of steroidogenesis. MA-10 mouse Leydig tumor cells were treated with and without oligodeoxynucleotides (ODNs) antisense to PBR and StAR followed by treatment with saturating concentrations of human choriogonadotropin. Treatment with ODNs antisense but not missense for both proteins inhibited the respective protein expression and the ability of the cells to synthesize steroids in response to human choriogonadotropin. Treatment of the cells with either ODNs antisense to PBR or a transducible peptide antagonist to PBR resulted in inhibition of the accumulation of the mature mitochondrial 30-kDa StAR protein, suggesting that the presence of PBR is required for StAR import into mitochondria. Addition of *in vitro* transcribed/translated 37-kDa StAR or a fusion protein of Tom20 (translocase of outer membrane) and StAR (Tom/StAR) to mitochondria isolated from control cells increased pregnenolone formation. Mitochondria isolated from cells treated with ODNs antisense, but not missense, to PBR failed to form pregnenolone and respond to either StAR or Tom/StAR proteins. Reincorporation of *in vitro* transcribed/translated PBR, but not PBR missing the cholesterol-binding domain, into MA-10 mitochondria rescued the ability of the mitochondria to form steroids and the ability of the mitochondria to respond to StAR and Tom/StAR proteins. These data suggest that both StAR and PBR proteins are indispensable elements of the steroidogenic machinery and function in a coordinated manner to transfer cholesterol into mitochondria. (*Molecular Endocrinology* 19: 540–554, 2005)

STEROID BIOSYNTHESIS BEGINS with the transfer of free cholesterol from intracellular stores into mitochondria. The first enzymatic step in the steroidogenesis, the conversion of cholesterol to pregnenolone, is catalyzed by the cytochrome P450 side chain cleavage (P450scc) present in the inner mitochondrial membrane (IMM) (1–4). Pregnenolone then leaves the mitochondrion to undergo enzymatic transformation in the endoplasmic reticulum where it can be metabolized to other steroid products. Thus, the amount of cholesterol available to P450scc for pregnenolone synthesis determines the amount of steroid formed. Considering the physicochemical characteristics of cholesterol and the route it has to take from intracellular stores to P450scc, there are four major steps involved in this cholesterol movement: 1) transfer of free cholesterol from the stores to the outer mitochondrial membrane (OMM); 2) loading of cholesterol into the OMM, where it remains separate from the structural cholesterol; 3) crossing from the OMM to IMM across the aqueous intermembranous space; and 4) loading onto the P450scc on the matrix side of the IMM (1–4). Among these, steps 1 and 3 have been shown to be the sites where peptide hormones and the second messenger cAMP act to accelerate cholesterol transfer and thus rapidly induce steroid hormone biosynthesis (1–4).

Two proteins appear to play a critical role in intramitochondrial cholesterol transport: the peripheral-type benzodiazepine receptor (PBR) (5) and the steroidogenic acute regulatory protein (StAR) (6). PBR is

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Abbreviations: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; hCG, human choriogonadotropin; IMM, inner mitochondrial membrane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ODN, oligodeoxynucleotide; OMM, outer mitochondrial membrane; PBR, peripheral-type benzodiazepine receptor; PKA, protein kinase A; P450scc, cytochrome P450 side chain cleavage (CYP11A); siRNA, short interfering RNA; StAR, steroidogenic acute regulatory protein; Tim, translocase inner membrane; Tom, translocase outer membrane.

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an 18-kDa protein originally discovered because it binds the benzodiazepine diazepam with relatively high affinity (5, 7). Although present in all tissues examined, PBR is particularly abundant in steroid-producing tissues, where it is localized primarily in the OMM (5, 7). In various cell systems and in isolated mitochondria, PBR drug ligands stimulate the formation of steroids (5, 8). Quantitation of the cholesterol present in the OMM and IMM before and after treatment with PBR ligands shows that PBR ligands induce cholesterol transfer from OMM to IMM (8, 9). Targeted disruption of the PBR gene in R2C Leydig cells arrested cholesterol transport into mitochondria and steroid formation; transfection of the PBR-disrupted cells with a PBR cDNA rescued steroidogenesis (10). Knocking out the PBR gene in mice resulted in embryonic lethal phenotype (8), suggesting a critical role of this protein. PBR, which is abundant in the outer/inner membrane contact sites (11), binds cholesterol to its cytosolic carboxy-terminal domain with high affinity (12–14). Although initial studies indicated that PBR may function as a channel for cholesterol (11), more recent studies suggest that it may also function as a steroid exchanger (8). Studies of PBR in various tissues indicate that it also participates in apoptosis (15) and tissue regeneration/recovery (16). The latter functions may also relate to its ability to import cholesterol into mitochondria, thus participating in mitochondrial membrane biogenesis.

StAR was initially described as a rapidly induced family of 37-, 32-, and 30-kDa phosphoproteins in ACTH-treated rat and mouse adrenocortical cells, and in LH-treated rat corpus luteum cells and mouse Leydig tumor cells (6). StAR is formed as a 37-kDa protein containing an N-terminal mitochondrial signal sequence (17), which is rapidly transported into mitochondria where it is cleaved, generating the inactive 32-kDa and 30-kDa intramitochondrial forms (18). StAR acts exclusively on the OMM (19, 20), and its import into mitochondria terminates its activity in steroidogenesis. The causative role of mutated inactive forms of StAR in congenital lipoid adrenal hyperplasia (21, 22) and the decreased steroid formation in mice where the StAR gene was knocked out (23) demonstrated the critical role of this protein in steroid biosynthesis. By homology with the x-ray crystal structure of the StAR-related lipid transfer domain of MLN64 (24), StAR can bind sterol and facilitate the transfer of cholesterol from sterol-rich unilammelar vesicles to acceptor membranes (25, 26) as well as stimulate steroidogenesis when used on isolated steroidogenic mitochondria (20). However, the importance of sterol binding to the StAR-related lipid transfer domain of StAR, as well as the interaction of StAR with the OMM, remains to be clarified. Although there is evidence that StAR can bind cholesterol at a 1:1 molar ratio (24), there also is *in vitro* evidence that the stoichiometry of transfer of cholesterol induced by StAR is 1.8 molecules of cholesterol per molecule of StAR (26) and *in vivo*, StAR can facilitate the transfer up to 400 molecules of cholesterol per min in adrenal cells (27), making the protein a very efficient inducer of cholesterol transport.

Although intramitochondrial 30-kDa StAR protein has been used as the main marker of hormone-induced steroid formation, it is now clear that StAR acts exclusively on the OMM (19, 20) where it undergoes a pH-dependent conformational change (28, 29) and that StAR import terminates its activity (25, 30). These studies further suggested that OMM components play a crucial role in mediating StAR activity and function (28, 29).

A recent study using fluorescence resonance energy transfer between StAR fused to enhanced green fluorescent protein and PBR fused to yellow fluorescent protein suggested that these two proteins may interact under physiological conditions (31) and suggested that StAR may function to bring cholesterol to the PBR in the OMM (31, 32). To clarify the functional relationships between PBR and StAR, we examined their roles in hormone-induced steroid synthesis and the role of PBR in StAR-mediated cholesterol transport and steroid formation. Our data demonstrate that both StAR and PBR are indispensable elements of the steroidogenic machinery and function in a coordinated manner to transfer cholesterol into mitochondria, and suggest that PBR is required for StAR and cholesterol import into mitochondria.

RESULTS

Effects of Human Choriogonadotropin (hCG) on MA-10 Leydig Cell Steroid Formation, 18-kDa PBR, and 30-kDa StAR Protein Levels

Under basal conditions, MA-10 cells make minimal amounts of progesterone (10–20 ng/mg protein) with minor changes in the amount of progesterone accumulated over time (Fig. 1A). Under these conditions, basal levels of 30-kDa StAR and 18-kDa PBR proteins are reduced over time in culture (Fig. 1B) in a significant manner ($P < 0.001$ by ANOVA). Treatment of MA-10 cells with saturating amounts of hCG (50 ng/ml) induced a dramatic time-dependent increase in progesterone formation (Fig. 1A), a modest but significant ($P < 0.001$ by ANOVA) increase in 30-kDa StAR protein levels, and no significant change in 18-kDa PBR levels (Fig. 1C). Presenting the protein expression data as the ratio of hCG/control treatment shows a significant 3-fold increase in StAR protein levels and minor 1.3-fold increase in PBR protein levels (Fig. 1D).

StAR Protein and a Benzodiazepine PBR Drug Ligand Stimulate Mitochondrial Pregnenolone Formation to the Same Extent but with Distinct Kinetics

In agreement with previous studies, StAR, made from a StAR construct expressed in an *in vitro* transcription/translation system, and the PBR-selective benzodiazepine ligand Ro5–4864, stimulated pregnenolone formation by isolated MA-10 mitochondria by 1.8- and

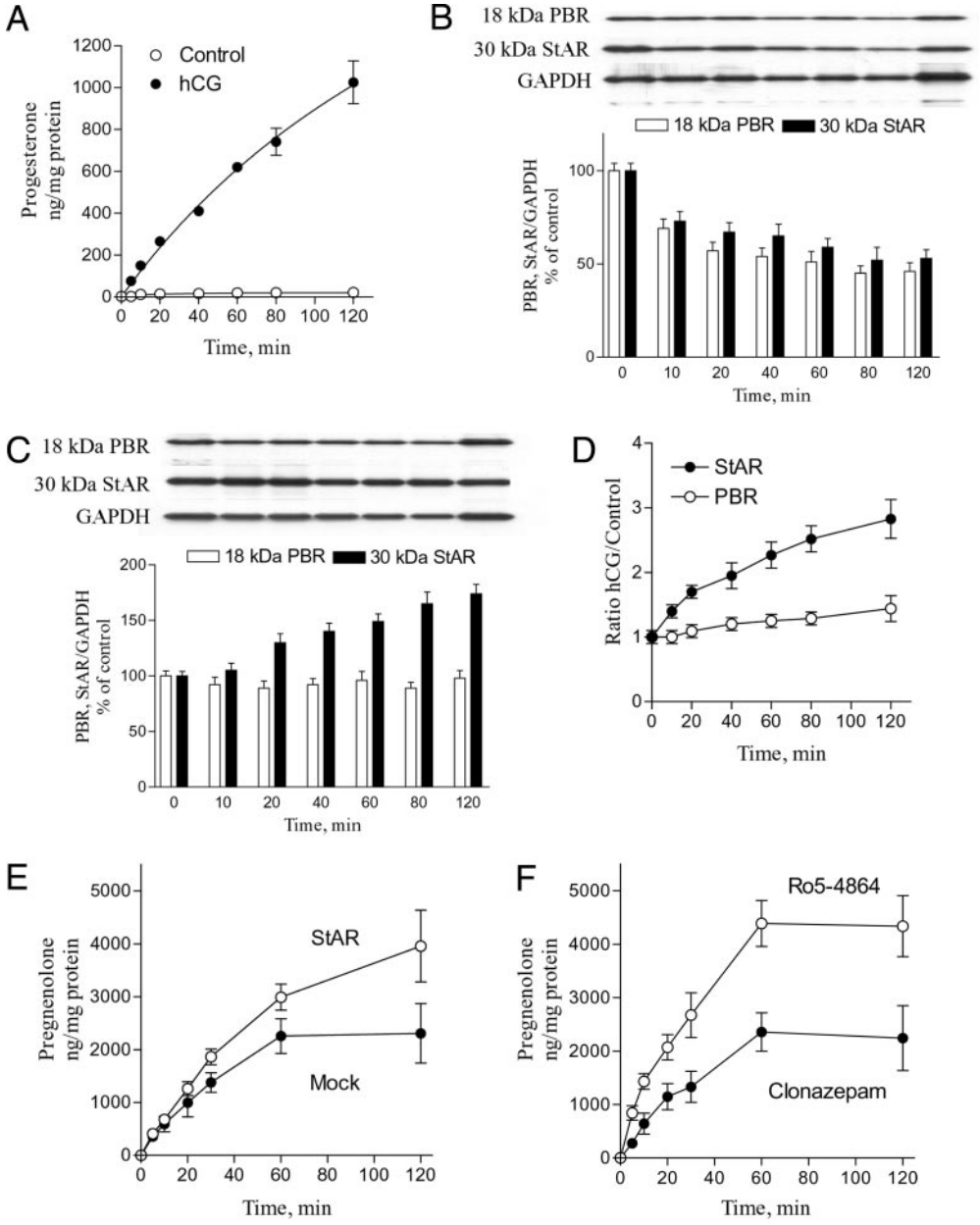

Fig. 1. Relationship of PBR and StAR Levels to Steroid Formation in MA-10 Cells

A, Time course of saturating amounts of hCG (50 ng/ml) on progesterone formation. Cells were treated for the indicated time points. Media were collected and progesterone levels were determined by RIA. Results are means ± SD from four independent experiments, each one conducted in triplicate. B, 18-kDa PBR and 30-kDa StAR protein levels in MA-10 cells. Cells were washed and incubated with serum-free media; at the indicated times, cell extracts were prepared, and proteins were separated by SDS-PAGE and identified by immunoblot analysis. The *upper panel* shows representative immunoblots for the 18-kDa PBR, 30-kDa StAR, and control GAPDH. The *lower panel* shows the ratio of PBR and 30-kDa StAR to GAPDH for each time point. Results shown are means ± SD from three independent experiments. C, PBR and 30-kDa StAR protein levels in hCG-treated MA-10 cells. Cells were washed and then incubated with 50 ng/ml hCG in serum-free media. At the indicated time points, cell extracts were prepared, and proteins were separated by SDS-PAGE and identified by immunoblot analysis. The *upper panel* shows representative immunoblots for the 18-kDa PBR, 30-kDa StAR, and control GAPDH. The *lower panel* shows the ratio of PBR and 30-kDa StAR to GAPDH for each time point. Results shown are means ± SD from three independent experiments. D, Effect of hCG on 30-kDa StAR and PBR protein levels. Data collected from all protein expression studies (panels B and C) were analyzed as ratios of hCG/control. Results shown are means ± SD from three independent experiments. E, Effect of full-length 37-kDa StAR on MA-10 mitochondrial pregnenolone formation. *In vitro* transcribed and translated 37-kDa StAR protein was incubated with isolated MA-10 mitochondria, and pregnenolone formation was determined by RIA at the indicated time points. Results shown are means ± SD from three independent experiments. F, Effect of PBR drug ligands Ro5–4864 (1 μM) and clonazepam (10 μM) on pregnenolone formation from isolated MA-10 cell mitochondria. Pregnenolone formation was determined by RIA at the indicated time points. Results shown are means ± SD from three independent experiments.

2-fold (Fig. 1, E and F). The respective controls, mock and the benzodiazepine clonazepam (which has no affinity for PBR), did not affect basal pregnenolone formation. Although pregnenolone synthesis in response to either StAR or PBR was of the same order of magnitude, the kinetics of the responses were different, showing a rapid (5 min) significant induction of mitochondrial steroid formation by Ro5–4864 (Fig. 1F) and a slower (30 min) induction of the response by StAR (Fig. 1E).

Treatment of Leydig Cells with Oligodeoxynucleotides (ODNs) Antisense to PBR or StAR Inhibit PBR and StAR Protein Expression and hCG-Stimulated Steroidogenesis

We used ODNs antisense to PBR and StAR to assess the impact of these proteins on Leydig cell function. Fluorescence microscopy demonstrated that fluorescein isothiocyanate-labeled ODNs were efficiently taken up by MA-10 cells; ODNs appeared in the cells within 12 h and remained stable for 72 h (data not shown). Antisense ODNs inhibited the expression of both PBR and StAR in a dose-dependent (Fig. 2, A and B) and significant ($P < 0.001$ by ANOVA) manner. Numerous experiments showed that 5 μM ODN was optimal for blocking 60% of StAR and PBR expression (Figs. 2A and 3). Inhibition of PBR and StAR protein expression was linked to inhibition of both basal and hCG-stimulated progesterone production (Fig. 3, C and D; $P < 0.001$ by ANOVA). ODNs missense for PBR and StAR had no effect on StAR, PBR, or steroid formation. Under both basal (Fig. 3, E and F) and hCG-stimulated (Fig. 3, G and H) conditions, the ODN treatment did not affect cell viability or mitochondrial integrity, as shown by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay based on the reduction of mitochondrial diaphorase (Fig. 3, E–H).

To assess the role of these proteins in the acute steroidogenic response, we examined the kinetics of hCG-induced steroid synthesis in PBR- and StAR-depleted MA-10 cells. PBR-depleted cells stopped producing progesterone 10 min after exposure to hCG whereas StAR-depleted cells produced progesterone for 20 min after hCG treatment (Fig. 4A), with no effect on cell viability (Fig. 4B). Figure 4D confirms that in these experiments the ODNs antisense to PBR and StAR inhibited the respective protein expression.

Inhibition of PBR and StAR Protein Expression Does Not Affect the hCG-Induced Protein Kinase A (PKA) Activity and 22*R*-Hydroxycholesterol-Supported Steroid Formation

Considering the effect of ODNs antisense to PBR and StAR on hCG-stimulated steroid formation, the effect

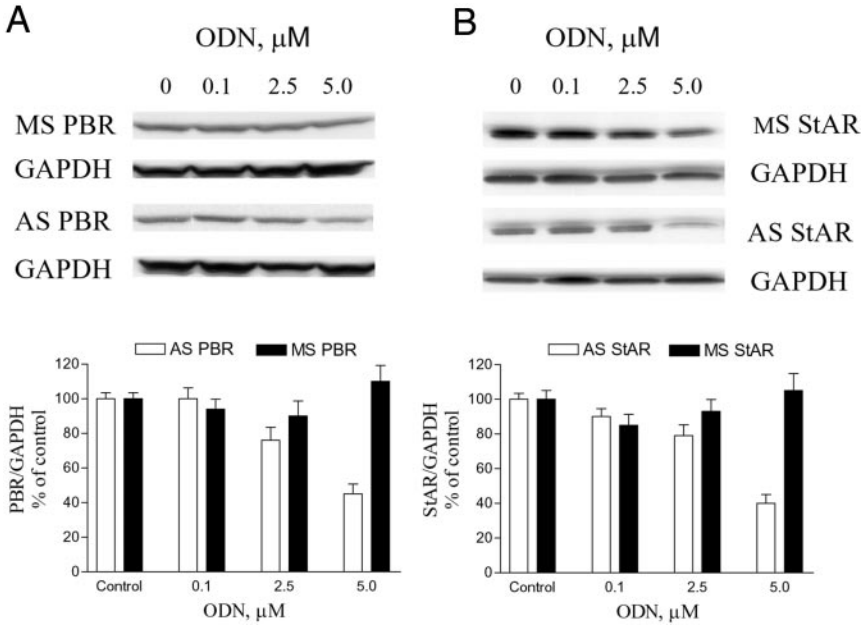

Fig. 2. Inhibition of PBR and StAR Protein Expression by ODNs Antisense to PBR and StAR

A, MA-10 cells were treated with the indicated concentration of ODNs antisense to PBR or missense control for 72 h. Proteins were separated by SDS-PAGE and identified by immunoblot analysis. *Upper panel* shows representative immunoblots for the 18-kDa PBR and the control GAPDH. The *lower panel* shows the ratio of PBR to GAPDH for each experimental condition. Results shown are means ± SD from three independent experiments. B, MA-10 Leydig cells were treated with the indicated concentration of ODNs antisense to StAR or missense control for 72 h, and proteins were separated by SDS-PAGE and identified by immunoblot analysis. The *upper panel* shows representative immunoblots for the 30-kDa StAR and the control GAPDH. The *lower panel* shows the ratio of 30-kDa StAR to GAPDH for each experimental condition. Results shown are means ± SD from three independent experiments.

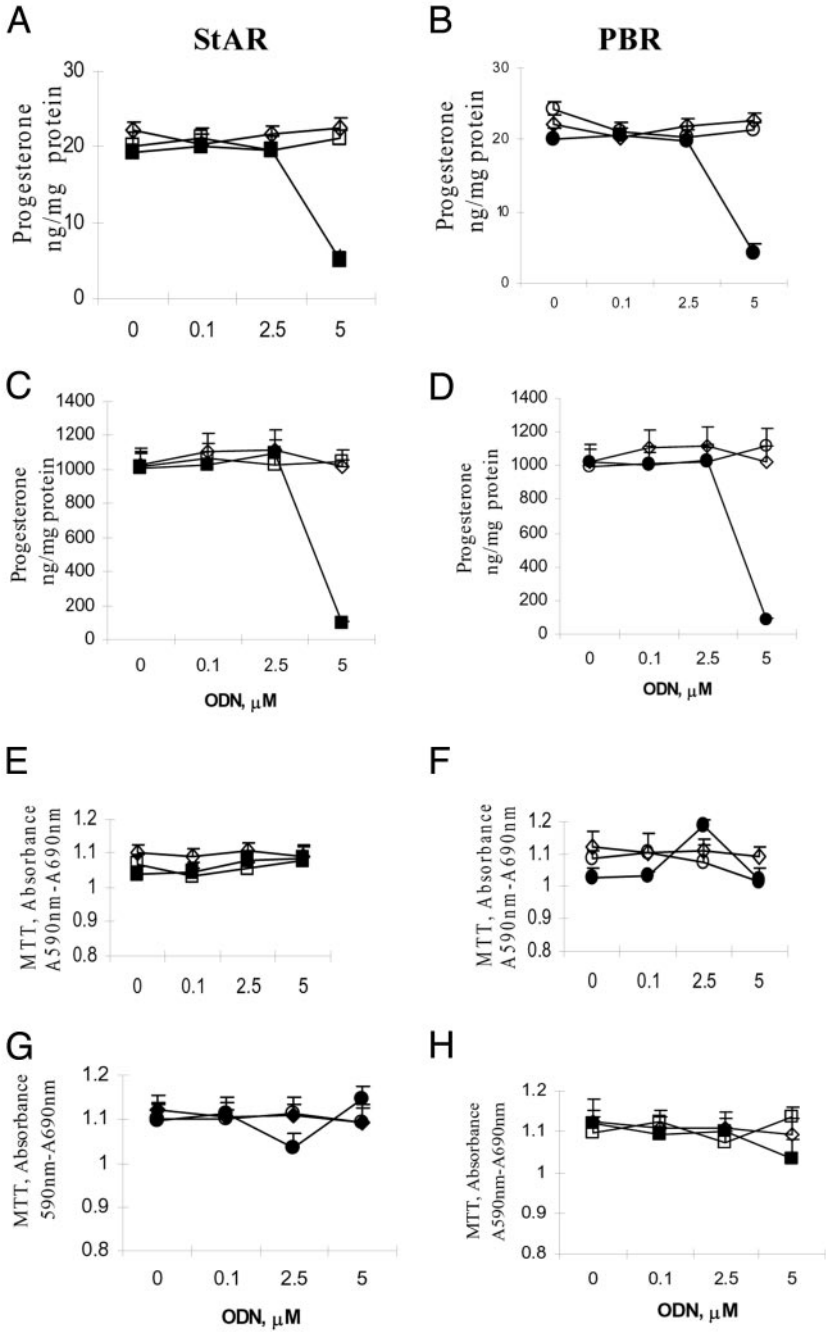

Fig. 3. Effect of ODNs Antisense to PBR and StAR on Basal and hCG-Induced Steroidogenesis

MA-10 cells were treated for 72 h without (◇), or with increasing concentrations of ODNs antisense (●, ■) or missense (○, □) to either StAR (A, C, E, and G) or PBR (B, D, F, and H). At the end of the treatment, cells were washed and treated without (A, B, E, and F) or with (C, D, G, and H) 50 ng/ml hCG. Progesterone levels were measured in the media by RIA (A–D). Results shown are means ± SD from four independent experiments (n =12). In separate experiments (E–H), cell viability was determined using the MTT assay (absorbance A_{600}–A_{690}). Results shown are means ± SD from three independent experiments, each conducted in triplicate.

of these ODNs on PKA activity was investigated. PKA activity was measured using a nonradioactive detection kit based on the PKA-specific substrate, PepTagA1 peptide (L-R-R-A-S-L-G). Figure 4C shows that the ODNs used, which inhibited the formation of the 18-kDa PBR and 30-kDa StAR (Fig. 4D), have no significant effect on the hCG-stimulated PKA activity.

The water-soluble P450scc substrate 22*R*-hydroxycholesterol induced a dramatic increase in progesterone formation (Fig. 4E). Treatment of the cells with

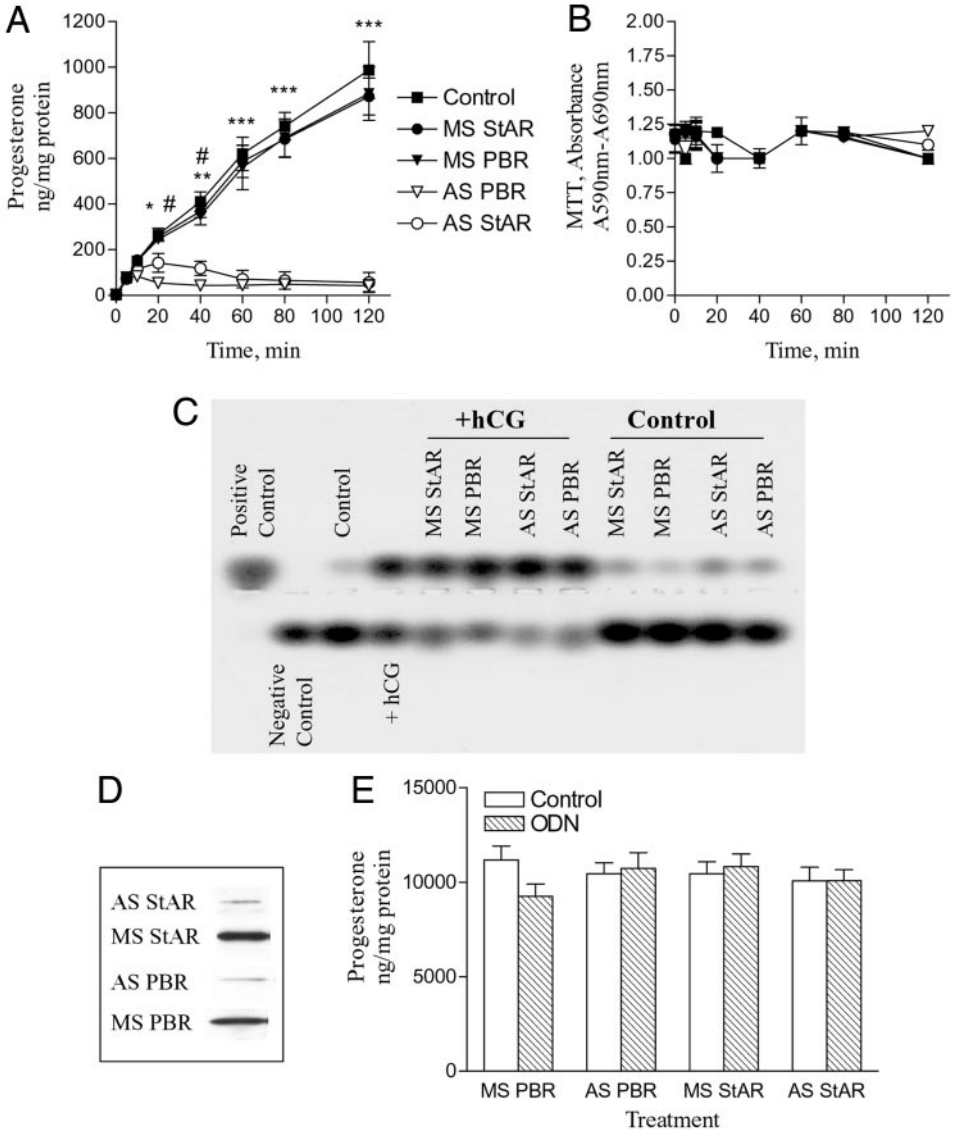

Fig. 4. Effect of ODNs Antisense to PBR and StAR on hCG-Induced Steroidogenesis

A, MA-10 cells were treated for 72 h with increasing concentrations of ODNs antisense (AS) or missense (MS) controls to either StAR or PBR. At the end of the treatment, cells were washed and treated with 50 ng/ml hCG for the indicated time periods. At each time period media were collected and assayed for progesterone levels by RIA. #, $P < 0.05$ ODN antisense to StAR *vs.* ODN antisense to PBR; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for ODNs antisense to PBR and StAR compared with their respective missense control ODN treatments. B, In separate experiments, cell viability was determined by MTT assay (absorbance A_{600}–A_{690}). Results shown are means ± SD from three independent experiments, each conducted in triplicate. C, Effect of the ODNs used on hCG-induced PKA activity. MA-10 cells were treated with 5 μM ODNs for 72 h followed by treatment with 50 ng/ml hCG for 60 min, and PKA activity was determined using the PepTag assay. D, PBR and StAR levels in MA-10 Leydig cells treated with 5 μM ODNs for 72 h. Proteins were separated by SDS-PAGE and identified by immunoblot analysis. E, Effect of the ODNs tested on steroidogenesis using 22*R*-hydroxycholesterol as substrate. MA-10 cells were treated with 5 μM ODNs for 72 h followed by treatment with 20 μM 22*R*-hydroxycholesterol for 2 h. At the end of the incubation, progesterone in the media was measured by RIA. Results shown are means ± SD from three independent experiments, each conducted in triplicate.

ODNs missense or antisense to PBR and StAR, did not inhibit the effect of 22*R*-hydroxycholesterol on steroidogenesis (Fig. 4E). In addition, the ODNs used did not modify the expression of P450scc, as assessed by immunoblot analysis of cell extracts (data not shown).

Treatment with PBR Antisense ODNs and a Transducible Peptide PBR Antagonist Inhibit the Formation of 30-kDa StAR Protein

To investigate whether there is a functional interaction between PBR and StAR proteins, we examined the

expression of intramitochondrial 30-kDa StAR in hormone-treated PBR-depleted MA-10 cells, which do not make progesterone (Fig. 4A). The formation of intramitochondrial 30-kDa StAR decreased up to 70% in a dose-dependent fashion; at the same time, the levels of 37-kDa StAR increased (Fig. 5A), suggesting that PBR on the OMM might be involved in StAR import and processing. To confirm this observation, we used the PBR peptide antagonist STPHSTP transduced into the cells. We built a fusion having TAT protein transduction domain at the amino terminus, followed by two glycine residues (to permit free bond rotation), followed by the 7-mer antagonist at the carboxy terminus. This antagonist construct (TAT-STPHSTP) and the corresponding 7-mer STHEETS (TAT-STHEETS) control were used as described previously (33). Preincubation with TAT-STPHSTP inhibited MA-10 steroidogenesis (33) and 30-kDa StAR protein

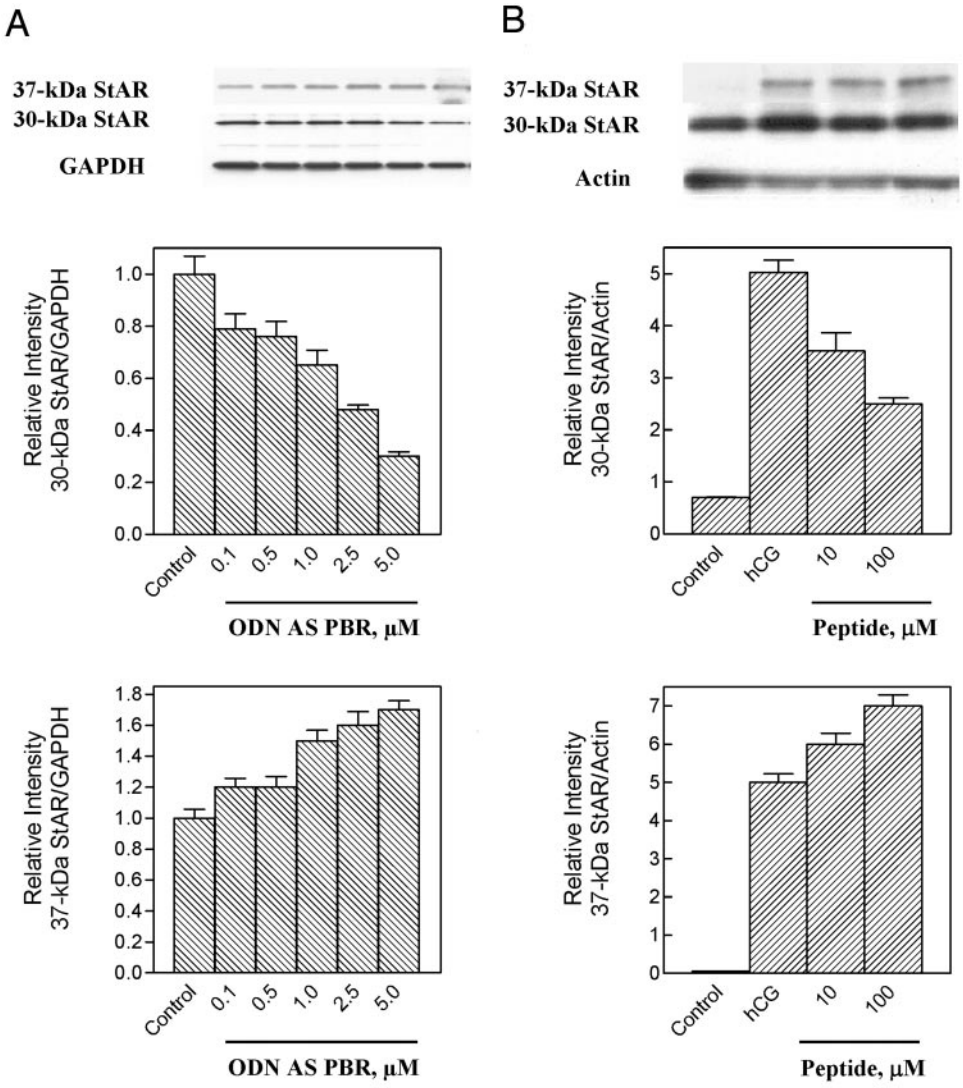

Fig. 5. Inhibition of PBR Expression or Function Blocks the Accumulation of Intramitochondrial 30-kDa StAR Protein

Left, MA-10 cells were treated with the indicated concentrations of ODNs antisense to PBR for 72 h and then exposed to 50 ng/ml hCG for 2 h. Proteins were separated by SDS-PAGE, and StAR was identified by immunoblot analysis. *Upper panel* shows representative immunoblots for the 30- and 37-kDa StAR and the control GAPDH. To visualize the 37-kDa StAR protein, the blot was exposed for longer periods of time. The *lower panel*s show the ratios of 30- and 37-kDa StAR to GAPDH for each experimental condition. Results shown are means ± SD from three independent experiments. *Right*, MA-10 cells were treated with the indicated concentration of TAT-STPHSTP for 30 min and then exposed to 50 ng/ml hCG for 2 h. Proteins were separated by SDS-PAGE, and 30-kDa StAR was identified by immunoblot analysis. The *upper panel* shows representative immunoblots for the 30-kDa and 37-kDa StAR and the actin control. To visualize the 37-kDa StAR protein, the blot was exposed for longer periods of time. The *lower panel* shows the ratios of 30- and 37-kDa StAR to actin for each experimental condition. Results shown are means ± SD from three independent experiments. ODN antisense to PBR and the PBR peptide antagonist TAT-STPHSTP inhibited the accumulation of 30-kDa StAR formation in a dose-dependent manner ($P < 0.001$ by ANOVA).

levels in a dose-dependent fashion (Fig. 5B), whereas the control peptide TAT-STHEETS did not affect hCG-induced 30-kDa StAR formation. In agreement with the data shown in Fig. 5A, 37-kDa StAR protein levels increased under these conditions. Thus, when the activity of PBR was blocked by the peptide antagonist, the import and processing of StAR, as assessed by the hormone-induced generation of intramitochondrial 30-kDa StAR protein, was partially blocked.

The effects on protein expression and steroid formation seen using ODNs antisense to PBR and StAR were replicated, although to a lesser extent, using RNA interference methodology to suppress the expression of their mRNAs (data not shown).

StAR, N-62 StAR, and Tom/StAR Stimulate Control But Not PBR-Depleted Mitochondrial Pregnenolone Synthesis

To examine the role of PBR in StAR import, we used *in vitro* transcribed/translated StAR in mitochondrial import assays. In agreement with previous studies (20), MA-10 mitochondria incubated with control cell-free transcription/translation system (mock) or that expressing Tim9/StAR (translocase inner membrane 9 StAR fusion) yielded minimal steroid production over 4 h (Fig. 6A). Expression of full-length StAR or N-62 StAR increased steroidogenesis 1.6-fold over control in the same time period. By contrast, expression of Tom/StAR (translocase outer membrane 20/StAR fusion) increased pregnenolone formation by the mitochondria 3-fold (Fig. 6A).

To examine the role of PBR in StAR import, we performed similar experiments using mitochondria from MA-10 cells treated with ODNs antisense (Fig. 6B) and missense (Fig. 6C) to PBR. PBR-depleted mitochondria made minimal amounts of pregnenolone and neither full-length StAR nor Tom/StAR induced pregnenolone formation (Fig. 6B). By contrast, mitochondria from cells treated with ODNs missense to PBR responded to StAR and Tom/StAR treatment (Fig. 6C) in the same way as control mitochondria. These studies suggest that the presence of PBR in the OMM is necessary for StAR action and import.

Reincorporation of *in Vitro* Translated/Transcribed PBR to PBR-Depleted Mitochondria Recovers, in Part, PBR Ligand-Binding and Steroid-Synthesizing Activities

To confirm the role of PBR in mediating StAR action and import into MA-10 cell mitochondria, we reincorporated PBR prepared by *in vitro* transcription/translation into PBR-depleted MA-10 mitochondria (Fig. 7A). As described previously (34), PBR forms polymers during this process. We previously showed that the cytosolic carboxy terminus of PBR and especially Y153 are critical for cholesterol binding (12, 14). Therefore, we examined the effects of deleting the amino terminus (N-10 PBR) or carboxy terminus (C-17 PBR) of PBR or replacing tyrosine 153 with serine (Y153S PBR). The various PBR proteins generated were of the correct size, and all of them were incorporated into mitochondria (Fig. 7A).

To assess how much of PBR was reincorporated into mitochondria and whether the reincorporated protein was correctly folded in its functional orientation, we performed radioligand binding studies in which the ability of the reincorporated PBR to bind PK11195 would indicate that the reincorporated PBR was functional. Treatment of MA-10 cells with ODNs antisense to PBR decreased PK11195 ligand binding capacity by 87.5% (Fig. 7B), in agreement with our data using immunoblot analysis (Fig. 4D). Incubation of mitochondria from cells treated with ODNs missense to PBR with *in vitro* transcribed/translated wild-type PBR in-

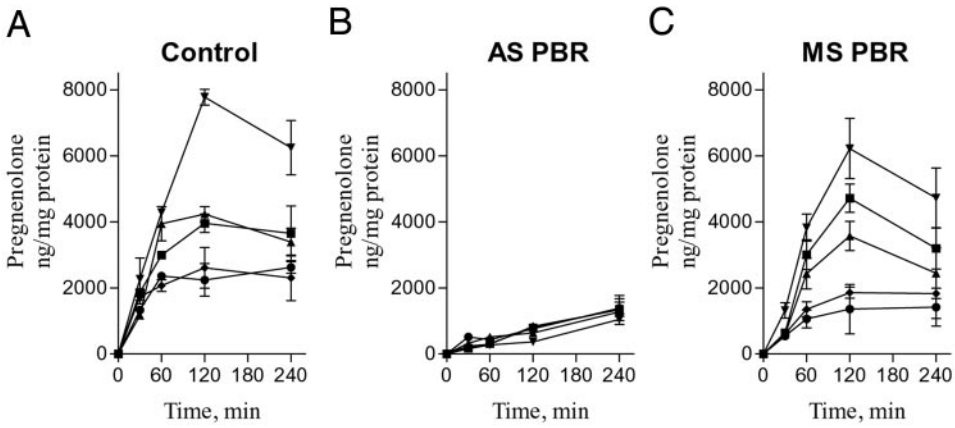

Fig. 6. StAR Does Not Stimulate Pregnenolone Synthesis in PBR-Depleted Mitochondria

MA-10 cells were treated for 72 h without (A) or with 5 μM ODNs antisense (B) or missense (C) to PBR. At the end of the incubation time mitochondria were isolated and incubated with *in vitro* transcribed and translated full-length 37 kDa StAR (▲), N-62 StAR (■), Tom/StAR (▼), Tim9/StAR (●), or mock (♦) and pregnenolone was determined by RIA at the indicated time points. Results shown are means ± SD from two independent experiments, each conducted in triplicate.

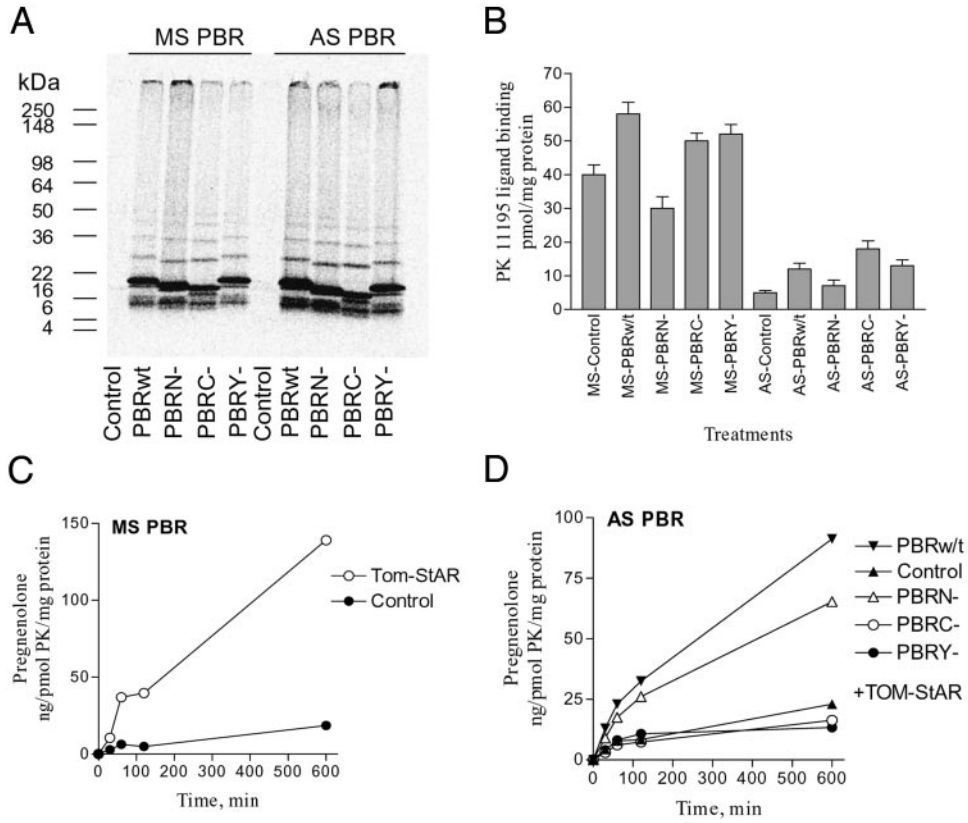

Fig. 7. Role of PBR in Mediating the Effect of Tom/StAR on Mitochondrial Pregnenolone Formation

A, Mouse wild-type PBR (PBR), N-10 PBR (PBRN−), C-20 PBR (PBRC−), and Y153S PBR (PBRY−) cDNAs were transcribed and translated *in vitro*, and the radiolabeled protein products were incubated for 30 min with Leydig cell mitochondria obtained from cells treated for 72 h with 5 μM ODNs antisense (AS) or missense (MS) to PBR. Mitochondrial proteins were separated by SDS-PAGE and visualized by phosphoimaging. B, PBR expression levels in mitochondria from MA-10 cells treated with ODNs antisense or missense to PBR measured by radioligand receptor assays using PK11195. C, MA-10 cells were treated for 72 h with 5 μM ODNs missense to PBR. Mitochondria were isolated and treated with *in vitro* transcribed and translated Tom/StAR (○) or mock (●). At the indicated time periods, media were collected and pregnenolone levels were determined by RIA. D, In the same experiments as C, mitochondria were incubated with *in vitro* transcribed and translated wild-type PBR (▼), N-10 PBR (Δ), C-20 PBR (○), Y153S PBR (●), or control mock (▲) for 30 min before the addition of *in vitro* transcribed/translated Tom/StAR. At the indicated time periods, media were collected and pregnenolone levels were determined by RIA.

creased PBR ligand binding capacity by 45% whereas reincorporation of wild-type PBR into PBR-depleted mitochondria increased in PBR ligand binding capacity by 240% (Fig. 7B). Reincorporation of C-17 PBR or Y153S PBR into PBR-depleted MA-10 mitochondria increased PK11195 binding capacity 3-fold, but reincorporation of N-10 PBR into PBR-depleted mitochondria increased in PK11195 binding capacity by only 40%, suggesting that the reinsertion of PBR into mitochondria may require an intact amino terminus.

The effect of the highly active Tom/StAR construct on pregnenolone formation by mitochondria isolated from MA-10 cells treated with ODNs missense to PBR and further incubated with wild-type PBR was then examined. The results are expressed per pmol of PK11195 bound per mg of mitochondrial protein (Fig. 7C). As expected, Tom/StAR stimulated mitochondrial pregnenolone formation by 5.8-fold and 7.5-fold over control in 2- and 10-h incubation time periods, respectively. In parallel studies, using PBR-depleted mitochondria, we first noted that Tom/StAR had no effect on pregnenolone synthesis (control; Fig. 7D), in agreement with the data shown in Fig. 6. However, reincorporation of wild-type PBR into mitochondria resulted in rescuing the response of the mitochondria to Tom/StAR and their steroidogenic ability as seen by the 4.5-fold stimulation of pregnenolone formation induced by Tom/StAR. Reincorporation of N-10 PBR also rescued the ability of the mitochondria to respond to Tom/StAR, although to a lesser extent, as shown by the 3.2-fold increase in pregnenolone formation induced by Tom/StAR. In contrast, reincorporation of C-17 PBR and Y153S PBR failed to rescue the ability of the mitochondria to respond to Tom/StAR (Fig. 7D).

It should be noted than when a StAR construct was used instead of Tom/StAR in the *in vitro* reconstitution assays, similar results were observed (data not shown) except that the effect of StAR on pregnenolone formation was much less pronounced (2-fold) than that of Tom/StAR (6-fold).

DISCUSSION

Cholesterol transport into mitochondria is the first, rate-determining, and hormone-dependent step in steroidogenesis (1–4). The search for factors involved in this transport identified PBR and StAR, but it was not clear whether these proteins interacted or how they functioned, although one recent study suggested that StAR and PBR interact (31). Therefore we sought to assess the respective roles of PBR and StAR and their putative interaction in hormone-induced steroid formation in MA-10 mouse Leydig tumor cells.

MA-10 cells express StAR and process it to a 30-kDa intramitochondrial form (35) and also express high levels of PBR (36). Over time in culture, the basal levels of both StAR and PBR proteins decrease. Treatment with saturating amounts of hCG induced a dramatic time-dependent increase in progesterone formation parallel to increased StAR protein, seen as early as 10–20 min after initiation of the hormone treatment. We chose to use hCG instead of cAMP because hCG reflects better the *in vivo* situation in which the peptide hormone may affect steroidogenesis via a number of pathways that may not be accounted for when using cAMP analogs. This may explain the difference in the responses between hCG and cAMP, such as the lack of lag period in steroid increase that we did not see in our studies. A 3-fold increase in processed, intramitochondrial 30-kDa StAR was observed after 2 h of treatment with hCG. This increase probably reflects both the inhibition of StAR degradation, as seen in basal conditions, and novel StAR synthesis. These data agree with previous findings showing that StAR is formed as a 37-kDa protein, which is rapidly transported into mitochondria where it is cleaved, generating the inactive 30-kDa intramitochondrial StAR protein (20). Newly synthesized StAR mRNA is seen 30–60 min after stimulation (27) and is used to maintain increased amounts of StAR protein. The 3-fold increase in 30-kDa StAR levels that we observed in response to hCG are much lower than those previously reported in MA-10 cells (17, 35) and may be due to differences in cell culture or the sensitivity of the antisera used. Under the same conditions, there was no significant increase in the 18-kDa PBR levels, in agreement with previous findings (36). Nevertheless, although these studies focused on the fate and role of the 18-kDa PBR monomer, we recently reported that, in the same time frame, there is a hormone-induced PBR polymer formation (34, 37), leading to the formation of PBR dimers, trimers, and tetramers with higher binding capacity for cholesterol (34).

Effects of recombinant StAR, StAR transcribed and translated *in vitro*, and PBR drug ligands on isolated mitochondria have been reported previously, showing that all increase steroidogenesis (8, 19, 20, 26). Our data indicated that StAR and the PBR-selective benzodiazepine ligand Ro5–4864 stimulated pregnenolone formation from isolated MA-10 mitochondria by 1.8- to 2-fold. However, the kinetics of these responses differed: Ro5–4864 induced a rapid (5 min) induction of mitochondrial steroid formation, and StAR induced a slower (30 min) induction. The basis for this difference is not clear.

To assess the respective impact of PBR and StAR on Leydig cell function, we used antisense ODNs. Antisense, but not missense, ODNs inhibited expression of both PBR and 30-kDa StAR, and both basal and hCG-stimulated progesterone production with similar efficacy. The inhibition of StAR and PBR paralleled the inhibition of steroid formation although, on average, the inhibition of steroid formation by ODNs to PBR and StAR was greater that the percent inhibition of PBR and StAR levels. This observation suggests that either there is a minimal amount of PBR and StAR required to maintain steroid formation or that not all PBR and StAR in the cell are used for steroid synthesis. For StAR, measurement of the 30-kDa mature protein levels might not reflect steroidogenic activity because activity ceases upon cleavage of the 37-kDa StAR protein (18–20, 27).

To assess the roles of StAR and PBR in the acute steroidogenic response, we examined the kinetics of hCG-induced steroid synthesis in MA-10 cells depleted of PBR or StAR. After exposure to hCG, PBR-depleted cells stopped producing progesterone sooner (10 min) than StAR-depleted cells (20 min). Steroidogenesis in PBR-depleted cells during the first 10 min might reflect cholesterol already present in the OMM or contact sites, which would be thus available for steroidogenesis. Steroidogenesis in StAR-depleted cells may be due to StAR-independent cholesterol transport (22), which is present during the first 20–40 min after hormone stimulation (38). In both cases, the initial steroid formation may be due to residual PBR and StAR protein expression, because the ODN-induced inhibition of protein expression was, on average, 60% and, at best, 90%. It is unlikely that new RNA and protein synthesis occurs during this time period to account for residual steroidogenesis, because separate studies in which cells were treated with hCG in the continuous presence of ODNs showed similar results (data not shown).

Treating the cells with ODNs missense for PBR and StAR had no effect on PBR, StAR, or steroid formation. Moreover, the ODN treatments did not affect cell viability, hCG-stimulated PKA activity, or 22*R*-hydroxycholesterol-supported steroidogenesis, suggesting that, as expected, decreased PBR and StAR expression inhibited the availability of cholesterol substrate for P450scc.

RNA interference has emerged as an important functional genomics tool for *in vivo* depletion of a gene product (39). To validate the results obtained with ODNs antisense to PBR and StAR, we repeated these experiments using small interfering RNAs (siRNAs) to inhibit the expression of these gene products. Although the results confirmed those obtained with

ODNs, siRNA failed to induce a robust inhibition of PBR and StAR protein expression (data not shown), even though using the same technology we were able to inhibit PBR expression by 80% in human cancer cells (40). Thus ODN technology appears to remain superior for PBR and StAR in mouse Leydig MA-10 cells.

Although the absence of StAR did not affect PBR protein expression (data not shown), the accumulation of intramitochondrial 30-kDa StAR protein was reduced significantly in PBR-depleted cells treated with hCG, and at the same time the levels of the precursor 37-kDa StAR protein increased, suggesting that PBR might be involved in StAR import and processing. These data are consistent with results using the PBR peptide antagonist STPHSTP transduced into the cells (33). The potential role of PBR in mitochondrial protein processing was first proposed by Wright and Reichenbecher (41), who showed that PBR drug ligands enhanced mitochondrial processing of the manganese-dependent superoxide dismutase, suggesting a role of PBR in the regulation of mitochondrial import of proteins. Our finding that the absence of PBR limits the hormone-induced increase in StAR import and processing further support a role of PBR in mitochondrial import. These data were corroborated by *in vitro* reconstitution studies using *in vitro* transcribed/translated StAR, N-62 StAR, Tim9/StAR, and Tom/StAR proteins in mitochondrial import assays (20). Expression of full-length StAR or N-62 StAR increased steroidogenesis by 1.6-fold over control, and expression of Tom/StAR, but not Tim9/StAR, fusion protein resulted in the maximal (3-fold) increase in pregnenolone formation by the mitochondria. Pregnenolone formation under these conditions reflects the amount of cholesterol (exogenous and endogenous) converted to pregnenolone rather than the rate of steroid formation. The observation that mitochondria incubated with N62-StAR and StAR exhaust their ability to make pregnenolone after 60 min incubation with cholesterol may be due either to the fact that not enough N-62 StAR protein may reach the mitochondria or that mitochondrial processing of N-62 StAR and StAR terminates their activity (19, 20). In contrast, mitochondria incubated with Tom/StAR continued to form pregnenolone, indicating that anchoring/immobilizing Tom/StAR to OMM provides for a continuous transfer of cholesterol into mitochondria. In conclusion, these findings confirm previous data indicating that the presence of StAR at the OMM is critical for its activity (20). As with the whole-cell studies, PBR-depleted mitochondria made minimal amounts of pregnenolone and failed to respond to StAR, N-62 StAR, and Tom/StAR. These data suggest that PBR is 1) the anchor site of StAR at the OMM where the protein binds to exert its action before being imported into mitochondria; or 2) part of a protein complex or mitochondrial domain where StAR acts to exert its stimulatory effect; or 3) it is part of the mitochondrial protein import machinery, thus controlling protein import into mitochondria in a nondiscriminatory manner. Although suggestion 3 is supported by previous findings on the effects of PBR ligands on manganese-dependent superoxide dismutase protein import into mitochondria (41) and by the fact that similar results were observed when either StAR or Tom/StAR were used, further studies are required to determine the role of PBR in protein import into mitochondria and whether it is part of the Tom complex (42).

Reincorporation of *in vitro* transcribed and translated wild-type PBR and N-10 PBR into PBR-depleted Leydig cell mitochondria rescued mitochondrial responsiveness to Tom/StAR and StAR, further suggesting a role for PBR in mediating the action of StAR, import into mitochondria, and subsequent steroid formation. Because PBR is a highly hydrophobic protein that may not reincorporate in the correct orientation and folding, we estimated the amount of correctly reincorporated and folded PBR by assessing its ability to bind PK11195. These studies suggested that at least part of the amino terminus of PBR might be required for the reincorporation of functional PBR into mitochondria in the *in vitro* system used herein, although this observation must be confirmed in further studies. The finding that C-17 PBR and Y153S PBR failed to rescue the ability of the mitochondria to respond to Tom/StAR suggests that either the cytosolic carboxy-terminal domain of PBR is critical for the functional interaction with StAR or its absence does not allow cholesterol to be transferred into mitochondria. Indeed, we previously reported that the cytosolic carboxy terminus of PBR and, more specifically, Y153 are critical for cholesterol binding to PBR (12, 14).

The inhibitory effect of ODNs antisense to PBR and PBR peptide antagonist on MA-10 Leydig cell intramitochondrial 30-kDa StAR protein levels and hormone-stimulated steroid synthesis resembles that observed using the protonophore carbonyl cyanide 3-chlorophenylhydrazone, a mitochondrial toxin (43, 44). These observations could suggest that decrease in the OMM PBR levels may result in loss of the mitochondrial electrochemical gradient, which would be responsible for the decreased StAR import and steroidogenesis. However, the finding that reincorporation of PBR into the PBR-depleted mitochondria rescued the StAR-induced mitochondrial steroid formation argues against a nonspecific mitochondrial toxicity, as that induced by carbonyl cyanide 3-chlorophenylhydrazone. Nevertheless, these data do not exclude the possibility that the presence of PBR may be linked to the maintenance of the mitochondrial electrochemical gradient and that a decrease in PBR levels may lead to a reversible loss of this gradient.

Thus, we find that PBR and StAR interact in the transfer of cholesterol across the OMM to the IMM. This is the first report that there is a functional interaction between StAR and PBR required for cholesterol delivery into mitochondria and subsequent steroid formation. Considering 1) the requirement for PBR and StAR for steroidogenesis, 2) the location of PBR in the

OMM, 3) that PBR is a ubiquitous mitochondrial structural protein, 4) the hormone-dependence and lability of StAR, and 5) the apparent role of PBR in StAR import into mitochondria, we hypothesize that PBR serves as a gate keeper in protein and cholesterol import into mitochondria, and that StAR serves the role of the hormone-induced activator, thus placing these two indispensable components of the steroidogenic machinery in their rightful place.

MATERIALS AND METHODS

MA-10 Leydig Cell Culture and Mitochondria Preparation

MA-10 mouse Leydig tumor cells were a gift from Dr. Mario Ascoli (University of Iowa). Cells were cultured in 75 cm^2-cell culture flasks (Dow Corning Corp., Corning, NY) and were grown in DMEM nutrient mixture F-12 Ham (Sigma Chemical Co., St. Louis, MO) supplemented with 5% fetal bovine serum and 2.5% heat-inactivated horse serum (Life Technologies, Gaithersburg, MD). Mitochondria were isolated by differential centrifugation as we previously described (9).

Treatment with Oligonucelotides (ODNs)

Antisense ODNs and controls (missense and fluorescein isothiocyanate-labeled ODNs) directed to PBR and StAR were designed and manufactured by Biognostik (Berlin, Germany). Antisense ODNs were the reverse complements of target sequences described in mouse PBR (45) and StAR (17), and no other cross-homologies were found in GenBank databases. No cross-homologies were found in GenBank for the missense ODNs used. Initial experiments were performed to study the dose effect of antisense ODNs for PBR (GCG CCA TAC ATA GTA G; lot no. A4343.8) and StAR (CAT TTG GGT TCC ACT C; lot no. A4341.3) and randomized mismatch controls with the same AT/CG ratio missense oligonucleotide for PBR (ATG CCT CTG TCT TCT G; lot no. C4167.5) and StAR (GCT CTA TGA CTC CCA G; lot no. C4249.1). The results obtained were confirmed using a separate set of antisense ODNs for PBR (GCA TAC CAC CGG AG; lot no. A4344.9) and StAR (GTT GTT CTT CCT GAG; lot no. A4342.8) and separate randomized mismatch controls for PBR (ACC GAC CGA CGT GT; lot no. C4117.1) and StAR (GTC CCT ATA CGA AC; lot no. C3763.8). Based on this observation, antisense ODNs for PBR 5′-GCG CCA TAC ATA GTA G-3′ and StAR 5′-CAT TTG GGT TCC ACT C-3′ as well as the randomized mismatch controls 5′-ATG CCT CTG TCT TCT G-3′ for PBR and 5′-GCT CTA TGA CTC CCA G-3′ for StAR were used.

Cells were treated for 3 d with 0.1, 1, 2.5, and 5 μM of each antisense and missense. At the end of incubation, cells were washed and treated for 2 h with hCG (50 ng/ml). Purified hCG (batch CR-125 of biological potency 11,900 IU/mg) was a gift from the National Hormone and Pituitary Program, NIH (Rockville, MD). In subsequent studies, a time course study was conducted with 1 μM and 5 μM of each antisense and missense. Cells were treated for 3 d with 0.1, 1, 2.5, and 5 μM of each antisense and missense. At the end of incubation, cells were washed and treated with hCG (50 ng/ml) for 0, 5, 10, 20, 40, 60, 80, and 120 min. In separate studies, the effect of antisense and randomized mismatch control ODNs was also studied in combination with a 22*R*-hydroxycholesterol stimulation. Cells were treated for 3 d with 0.1, 1, 2.5, and 5 μM of each antisense and missense. At the end of the incubation, cells were washed and treated with 22*R*-hydroxycholesterol (10 μM) for 2 h. To check the uptake and localization of ODNs, fluorescein isothiocyanate-labeled antisense ODNs for PBR and StAR were used. MA-10 cells were plated into 96-well plates at the density of 2.5×10^4 cells per well and incubated for 10, 20, 40, 60, 80, 120 min and 1, 2, and 3 d time period with a fluorescence-labeled ODN. Slides were viewed and pictures taken using a Olympus BX-40 microscope equipped with a PM20 camera system (Olympus Corp., Melville, NY).

Peptide Transduction into Cells

20-mer TAT-STPHSTP and TAT-STHEETP peptides were synthesized so that they contained an NH_2-terminal 11-mer TAT protein transduction domain (single-letter code, YGRKKRRQRRR) followed by two glycine residues (33). Peptides were synthesized by Bethyl Laboratories, Inc. (Montgomery, TX). Transduction experiments were performed as we previously described (14, 33). To determine the efficiency of TAT peptide incorporation into the cells, MA-10 cells were cultured overnight on eight-chambered SuperCell Culture Slides (Fisher Scientific, Pittsburgh, PA) at a concentration of approximately 25,000 cells per chamber. Media were replaced 24 h later with fresh media, and cells were treated with various concentrations of Oregon Green 488-labeled peptides for various time periods. After the incubation period, cells were washed with PBS and examined by fluorescent microscopy as described above.

siRNA Preparation and Transfection

Silencer siRNAs to mouse PBR and StAR genes were purchased from Ambion, Inc. (Austin, TX). The following pair gene-specific sequences were used: antisense small interfering PBR oligonucleotide template: 5′-CUGUGAAACCUCCCAGCUCtt-3′; sense small interfering PBR oligonucleotide template: 5′-GAGCUGGGAGGUUUCACAGtt-3′; antisense small interfering StAR oligonucleotide template: 5′-CAGCUUGGUGCCUUAAUCCtt-3′; sense small interfering StAR oligonucleotide template: 5′-GGAUUAAGGCACCAAGCUGtt-3′. siRNA preparation and purification were performed according to the manufacturer's instruction (Ambion, Inc.). These sequences are located in exon 2 and exon 1 of the mouse PBR and StAR genes, respectively. As control, the *Silencer* negative control no. 1 siRNA (Ambion) was used. Single transfections of 100 nM siRNA duplexes were performed using Oligofectamine Reagent (Invitrogen, San Diego, CA). Cells were treated with siRNAs for 24 h and assayed for silencing 3 d after transfection. At the end of the treatment, cells were washed and incubated with or without saturating concentrations of hCG (50 ng/ml) for 2 h. The effect of the treatment on PBR and StAR levels and hCG-induced steroid formation was assessed by immunoblot analysis and RIA, respectively.

Steroid Biosynthesis

For steroid synthesis experiments, MA-10 cells were plated into 96-well plates at 2.5×10^4 cells per well. Medium was replaced 24 h later with fresh medium, and cells were treated with the indicated concentrations of ODNs or peptides as described above. Cells were then stimulated with 50 ng/ml hCG or 20 μM 22*R*-hydrocholesterol in serum-free media for 2 h. 22*R*-hydrocholesterol was prepared as an ethanolic stock solution and used at a final ethanol concentration of 0.002% in media. Controls contained the same amount of ethanol. At the end of the incubation, culture media were collected and tested for progesterone production by RIA using antiprogesterone antisera (ICN Biochemicals, Inc., Costa Mesa, CA), following the conditions recommended by the manufacturer. [1,2,6,7-N-^{3}H]Progesterone (SA, 94.1 Ci/mmol) was obtained from NEN Life Science Products (Bos-

ton, MA). Progesterone production was normalized for the amount of protein in each well. RIA data were analyzed using MultiCalc software (EG&G Wallac, Inc., Gaithersburg, MD).

Analysis of Mitochondrial Integrity/Cell Viability

Cell viability at the end of the incubation protocols described above was assessed using the MTT assay for mitochondrial integrity (46) (Trevigen, Inc., Gaithersburg, MD). Formazan blue formation was quantified at 600 nm and 690 nm using the Victor quantitative detection spectrophotometer (EG&G Wallac) and the results were expressed as (OD_{600}–OD_{690}).

Immuno (Western) Blot Analysis

After treatment, MA-10 cells were washed with PBS, and sample buffer (25 mM Tris-HCl, pH 6.8; 1% sodium dodecyl sulfate; 5% β-mercaptoethanol; 1 mM EDTA; 4% glycerol; and 0.01% bromophenol blue) was added. Proteins were separated by electrophoresis onto a 4–20% SDS-PAGE gradient gel and electrophoretically transferred to a nitrocellulose membrane as previously described (14). Membranes were incubated with primary antibodies against PBR (1:2,500) (14), StAR (1:5,000) (27), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000; Trevigen, Inc.), or actin (1:1,000; neoMARKERS Inc., Fremont, CA). The 18-kDa PBR and 30-kDa StAR proteins were visualized using an ECL kit (Amersham Biosciences, Arlington Heights, IL) and horseradish peroxidase-goat antirabbit and horseradish peroxidase-rabbit antimouse secondary antisera used at 1:7,000 and 1:5,000 dilutions, respectively. To visualize the 37-kDa StAR, protein blots were exposed for longer periods of time. Image-densitometric analysis of the immunoreactive protein bands was performed using the OptiQuant-image analysis software (Packard BioScience, Meriden, CT).

cAMP-Dependent Protein Kinase (PKA) Measurement

Cells were cultured in six-well plates (2×10^5 cells per well) and treated as described above for steroid biosynthesis. At the end of the incubation, cells were washed twice with PBS and proteins were extracted using an extraction buffer (25 mM Tris-HCl, pH 7.4; 0.5 mM EDTA; 0.5 mM EGTA; 10 mM β-mercaptoethanol; 0.5 mM phenylmethylsulfonylfluoride; 1 μg/ml leupeptin; and 1 μg/ml aprotinin). After centrifugation at 18,500 $\times$ *g* for 15 min, the supernatants were kept for PKA activity assay. Samples were processed using the PepTag assay for nonradioactive detection of PKA activity following the manufacturer's recommendations (Promega Corp., Madison, WI).

Radioligand Binding Assays

[^{3}H]PK11195 [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinoline arboxamide] (SA, 85.5 Ci/mmol) was obtained from NEN Life Science Products. [^{3}H]PK11195 binding to 5 μg cell protein obtained from cells treated as described above, or 2 μg mitochondrial protein isolated from cells treated as described above, was performed as previously described (10, 12). Specific [^{3}H]PK11195 binding was analyzed using the iterative nonlinear curve-fitting program RadLig 4.0 (KELL suite, Biosoft, Cambridge, UK).

In Vitro Transcription and Translation of StAR, PBR, Fused, and Mutated Proteins

Human full-length StAR, N-62 StAR, Tom20/StAR, and Tim9/StAR constructs were prepared as previously described (20). Mouse wild-type PBR, N-10 PBR, C-17 PBR, and Y153S PBR constructs were prepared as previously described (12). All generated mutations and deletions were confirmed by sequencing. *In vitro* transcription and translation of the constructs was performed using the TNT Quick Coupled Transcription/Translation Systems (Promega Corp.) as previously described (34). In some experiments, *in vitro* transcription and translation of PBR and variants was performed in the presence of [^{35}S]methionine (SA, 1000 Ci/mmol; obtained from DuPont-New England Nuclear) following the manufacturer's recommendations. The product of the reaction was incubated for 30 min with MA-10 Leydig cell mitochondria isolated as previously described (9) from cells treated with ODNs antisense or missense to PBR. Mitochondria were washed twice, and proteins were separated by SDS-PAGE on a 4–20% gradient acrylamide-bis-acrylamide gel at 125 V for 2 h and transferred to nitrocellulose membrane. The membrane was exposed to a multipurpose phosphor screen for 4 h and analyzed by phosphoimaging using the Cyclone Storage Phosphor System (Packard BioScience), or overnight using x-ray film.

Mitochondrial Steroid Formation

Isolated mitochondria were suspended at a final protein concentration of 160 μg/ml in import buffer (125 mM sucrose, 1 mM ATP, 1 mM NADH, 50 mM KCl, 0.05 mM ADP, 2 mM DTT, 5 mM Na-succinate, 2 mM $Mg(OA)_2$, 2 mM KH_2PO_4, and 10 mM HEPES buffer at pH 7.5) supplemented with 50 pM cholesterol and 5 μM 3β-hydroxysteroid dehydrogenase inhibitor trilostane, a gift from Stegram Pharmaceuticals (Sussex, UK), in a 60 μl final volume (20). To this mixture 1 μl of the transcription/translation mix was added, and samples were incubated for the indicated time periods at 37 C. At the end of the incubation, samples were placed on ice and centrifuged, and supernatants were used to measure pregnenolone by RIA using antipregnenolone antiserum (ICN Biochemicals, Inc.), following the conditions recommended by the manufacturer. [7-*N*-^{3}H]Pregnenolone (SA, 25 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). Pregnenolone production was normalized by the amount of protein in each sample. RIA data were analyzed as described above. In separate experiments, mitochondria were incubated in import buffer supplemented with the transcription/translation mix, and protein incorporation into mitochondria was evaluated by immunoblot analysis.

Protein Measurement

Proteins were quantified using the dye-binding assay of Bradford (47) with BSA as the standard.

Statistical Analysis

Statistical analysis was performed by Student's *t* test and one-way ANOVA followed by the Student-Newman-Keuls test, using the Instat (version 3.00) package from GraphPad, Inc. (San Diego, CA).

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