Hormonal Regulation of Peripheral Benzodiazepine Receptor Binding Properties Is Mediated by Subunit Interaction†

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ABSTRACT: The peripheral benzodiazepine receptor (PBR) is composed of three subunits with molecular masses of 18, 30, and 32 kDa. Many physiological functions have been attributed to the PBR, including regulation of steroidogenesis. Furthermore, the PBR itself is under hormonal regulation. In the current study, we investigated the role of female gonadal sex hormones in the regulation of PBR expression in steroidogenic and nonsteroidogenic tissues. To accomplish this, adult female rats were pharmacologically castrated using chronic administration of the gonadotropin-releasing hormone agonist decapeptyl (triptorelin-D-Trp^6-LHRH). Half of these rats received 17β-estradiol as hormone replacement, while a control group received daily injections of vehicle only. We found that PBR binding capacity dropped by 40 and 48% in ovaries and adrenals, respectively, following decapeptyl administration, as opposed to no change in the kidney. This down-regulation of PBR densities was prevented by estradiol replacement. We did not find evidence for transcriptional, posttranscriptional, and translational mechanisms in this decapeptyl-induced down-regulation. In contrast, immunoprecipitation of the PBR complex, using antibodies against the 18- and 32-kDa subunits of the complex, demonstrated that there were changes in PBR subunit interactions, consistent with the down-regulation of PBR binding capacity. These findings represent a novel hormone-dependent posttranslational regulatory mechanism.

Peripheral benzodiazepine receptors (PBR)[1] were discovered in 1977 when rat peripheral tissues, used as negative controls for brain radioligand binding experiments, demonstrated high-affinity [^{3}H]diazepam binding (*1*). Since then, PBR have been found in most peripheral tissues (*2*, *3*) as well as in the central nervous system (*4*, *5*). This class of receptors exhibits high affinity for the benzodiazepine Ro 5−4864 and for the isoquinoline carboxamide PK 11195 (*6*, *7*) but low affinity for clonazepam. Purification and photoaffinity labeling studies have revealed that the PBR is a multimeric complex composed of three different proteins: an isoquinoline carboxamide-binding protein (IBP), a voltage-dependent anion channel (VDAC), and an adenine nucleotide carrier (ADC) (*8*). These PBR subunits have molecular masses of 18, 32, and 30 kDa, respectively.

Many physiological functions are associated with the PBR, including steroidogenesis, cell proliferation and differentiation, chemotaxis, and mitochondrial respiratory control (*9*−*12*); however, its main role is still unknown. Two important observations indicate that PBR are likely to play a role in steroidogenesis. First, PBR are found primarily on the outer mitochondrial membrane (*13*−*15*), and second, PBR are extremely abundant in steroidogenic organs (*3*, *5*). Furthermore, Papadopoulos and his colleagues (*12*) have data strongly suggesting that PBR play a major role in cholesterol transport across the mitochondrial membrane. Other investigators have demonstrated the PBR involvement in the regulation of steroid metabolism (*16*). Additionally, it has been shown that the steroidogenic acute regulatory protein (StAR) is involved in the acute trophic hormone regulation of steroid synthesis (*17*, *18*). Nevertheless, the mechanism of cholesterol transport into the mitochondrial matrix remains unclear, and it is quite likely that other proteins are also involved in this process. It has been shown that specific

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[1] Abbreviations: PBR, peripheral benzodiazepine receptor(s); IBP, isoquinoline carboxamide-binding protein; VDAC, voltage-dependent anion channel; ADC, adenine nucleotide carrier; StAR, steroidogenic acute regulatory protein; GnRH; gonadotropin-releasing hormone; E_2, estradiol; DBI, diazepam-binding inhibitor; ACTH, adrenocorticotropic hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

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ligands active at the PBR regulate mitochondrial steroidogenesis (*12*, *19*, *20*). Other studies have demonstrated that PBR expression is under hormonal regulation in male and female rats (*21*−*25*) as well as in Leydig tumor cell lines (*26*).

In the current study, we investigated the involvement of female gonadal sex hormones in the regulation of PBR expression in steroidogenic and nonsteroidogenic tissues. Furthermore, we evaluated the biochemical events occurring in this hormone-dependent regulatory process. To this end, we evaluated the impact of gonadotropin-releasing hormone (GnRH) agonist-induced ovarian suppression and 17β-estradiol (E_2) replacement on PBR expression in female rats.

MATERIALS AND METHODS

Animals. Female Sprague−Dawley rats (60 days old) were maintained under standard laboratory conditions of 12-h light/dark cycle at 24 °C. Food and water were available ad libitum. Rats ($n = 50$) were injected subcutaneously once daily for 21 days with the GnRH agonist decapeptyl (triptorelin-D-Trp^6-LHRH; Ferring, Malmö, Sweden), 250 μg/kg, a dose sufficient to induce ovarian suppression (*27*); half of these rats were simultaneously injected subcutaneously once daily for 21 days with E_2 (Sigma Chemical Co., St. Louis, MO), 250 μg/kg (*28*, *29*), while the other half were treated with E_2 vehicle (0.1 mL of sesame oil). A third group ($n = 25$) was injected with the vehicle only.

Twenty-four hours after the last injection the rats were sacrificed by decapitation, and peripheral organs were removed, dissected, frozen immediately in liquid nitrogen, and stored at −70 °C until assayed. All use and treatment of animals were approved by the local institutional review committee, following governmental guidelines.

Binding Assay. Ovaries, kidneys, and adrenal glands were thawed and homogenized in 50 volumes of 50 mM potassium phosphate buffer, pH 7.4, at 4 °C with a Brinkmann Polytron (setting 10) for 15 s. The homogenate was centrifuged at 49000*g* for 15 min, and the pellet (final concentration 75−500 μg/mL protein) was resuspended in the above-mentioned potassium phosphate buffer. Measurements of [^{3}H]PK 11195 binding were conducted as previously described (*16*). Binding assays contained 400 μL of cell membrane (30−200 μg of protein) and 25 μL of [^{3}H]PK 11195 (0.2−6.0 nM, final concentration) in the absence (total binding) or presence (nonspecific binding) of 75 μL of PK 11195 (10 μM, final concentration), up to a final volume of 500 μL. After incubation for 1 h at 4 °C, samples were filtered under vacuum over Whatman GF/C filters and washed three times with 5 mL of 5 mM potassium phosphate buffer, pH 7.4. Filters were placed in vials containing 5 mL of xylene-Lumax (3:1, v/v; Lumax was purchased from Lumac, Schaesberg, The Netherlands) and counted for radioactivity in a β-scintillation counter after 12 h.

Serum Progesterone Determination. Progesterone levels in the serum were determined to validate the efficacy of decapeptyl treatment. Coat-A-Count progesterone kit (DPC, Los Angeles, CA), a solid-phase radioimmunassay, was used to assess the hormone level. The inter- and intra-assay coefficients of variation were 5.7 and 4.8%, respectively.

Steady-State RNA Preparation and Analysis. Total RNA was isolated using Tri Reagent (Sigma). RNA (10−30 μg/lane), quantitated by absorbance at 260 nm, and fractionated by electrophoresis through 1% agarose/formaldehyde gels, and the fractionated RNA was transferred (in 20 × SSC) to 0.45 M nylon-reinforced nitrocellulose membranes (Nytran-N); Schleicher & Schuell, Dassel, Germany) by standard procedures (*30*). These blots were probed with [^{32}P]labeled cDNA probes (rat IBP or cyclophilin): the 750-bp *Eco*RI rat IBP cDNA fragment (a kind gift from Dr. Karl E. Krueger, Georgetown University, Washington, DC) and the 700-bp *Bam*HI-*Pst*I cyclophilin cDNA (*31*). Hybridizations and washes were carried out under standard conditions (hybridization at 65 °C; two washes with 1% SDS, and 2 × SSC at 65 °C for 20 min each). The blots were autoradiographed with a phosphor-imaging system (model BAS 1000, MacBas; Fuji, Tokyo, Japan) as well as multiple X-ray film exposures (Kodak XAR, Rochester, NY).

Nuclear Run-On Transcription Analysis. Replicate nitrocellulose membranes for hybridization were prepared in a slot-blot apparatus. Four micrograms of each plasmid DNA were blotted onto the membrane, as previously described (*32*, *33*). Nuclear run-on experiments were performed with our minor modifications (*34*) to those previously described (*32*, *33*).

Peptide Synthesis and Immunization. A peptide corresponding to a sequence of the exon-4 domain of the rat IBP subunit (CSTMLNYYVWRDNSGRRGGSRLTE) gene was synthesized (in the laboratory of Prof. Matti Fridkin, Weizmann Institute of Science, Rehovot, Israel). The synthetic peptide was conjugated to keyhole limpet hemocyanin (maleimide-activated KLH, Pierce Chemical Co., Rockford, IL) and injected subcutaneously into a rabbit every 3−4 weeks for several months. Blood was collected 1 week after each injection, and the antiserum was titrated by enzyme-linked immunosorbent assay using the synthetic peptide as coating antigen. This antiserum was used for Western blot analysis and for immunoprecipitation of the 18-kDa IBP−PBR subunit.

Isolation of Mitochondrial Fractions. Mitochondrial fractions were isolated as described previously (*13*), with minor modifications. The tissues (adrenals, kidneys, and ovaries) were incubated for 15 min in ice-cold buffer A [2 mM HEPES, 70 mM sucrose, and 210 mM D-mannitol, pH 7.4, and the "complete" protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany)]. About 1 g of tissue was homogenized in 2 mL of ice-cold buffer A, in a glass homogenizer, by four slow up-and-down strokes with a loose-fitting Teflon pestle. Then an additional 8 mL of ice cold-buffer A were added, and the homogenate was centrifuged for 15 min at 635*g* at 4 °C in the SM24 rotor of a refrigerated Sorvall RC-5B centrifuge. The supernatant was removed to a fresh test tube and centrifuged for 15 min at 6500*g* at 4 °C. The pellet resulting from this centrifugation was designated the "mitochondrial fraction." The mitochondrial pellet was gently homogenized in a final volume of 2 mL with ice-cold buffer A, and the mitochondrial suspension was centrifuged at 10000*g* for 15 min at 4 °C. The pellet was rehomogenized, this time in a final volume of 1 mL. After repeated centrifugation of the sample for 15 min at 10000*g*, the washed mitochondrial fraction was suspended in 1 mL of buffer A and stored at −70 °C until use.

Protein Measurements. Protein was quantitated by the method of Bradford (*35*) using BSA as a standard.

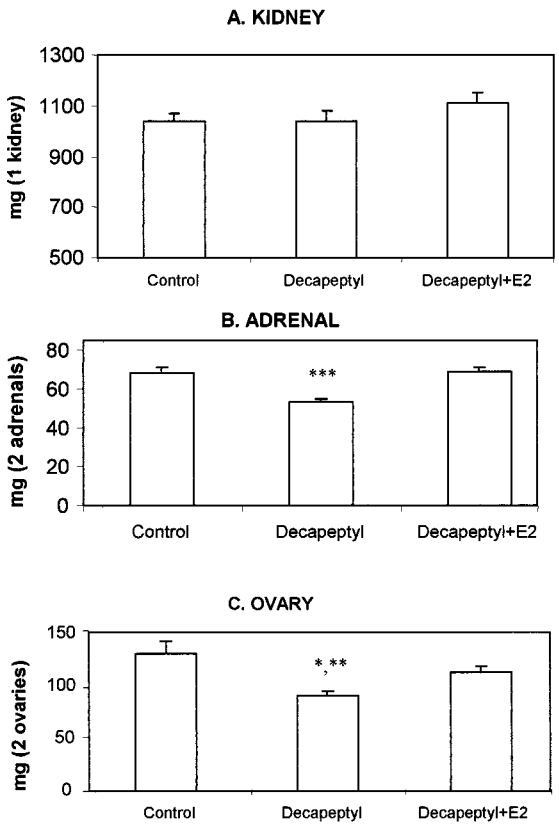

FIGURE 1: Tissue weight following pharmacological castration ($n = 6$ in each group). *$P < 0.05$ versus decapeptyl/E_2-treated rats; **$P < 0.001$ versus controls; ***$P < 0.001$ versus control and decapeptyl/E_2-treated rats.

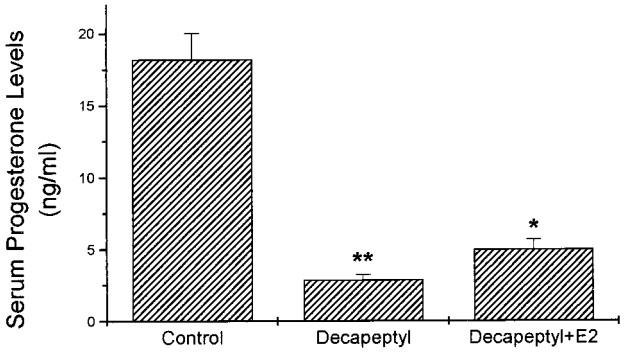

FIGURE 2: The impact of chronic decapeptyl administration and estradiol treatment on serum progesterone levels. E_2 = estradiol. **$P < 0.001$ versus control. *$P < 0.05$ versus control.

Western Blot Analysis. Mitochondrial fractions obtained from the different treated tissues were thawed, and SDS was added to a final concentration of 1%. After 10 min centrifugation of the sample at 10000*g*, the mitochondrial proteins were quantified and prepared in 2× sample buffer [0.125 M Tris-HCl (pH 6.8), 20% glycerol, 4% (w/v) SDS, 0.14 M β-mercaptoethanol, and 0.005% (w/v) bromphenol blue]. The samples were boiled for 10 min and subjected to electrophoresis through 4−20% SDS−polyacrylamide gradient gels (10−40 μg of protein/lane). The protein extracts were then electrophoretically transferred to nitrocellulose (Hybond ECL; Amersham Life Sciences, Buckinghamshire, England) in 20 mM Tris-HCl, 150 mM glycine, and 20% methanol for 1 h at 100 V, followed by blocking of the membrane in 5% dried milk (Carnation, Glendale, CA) in

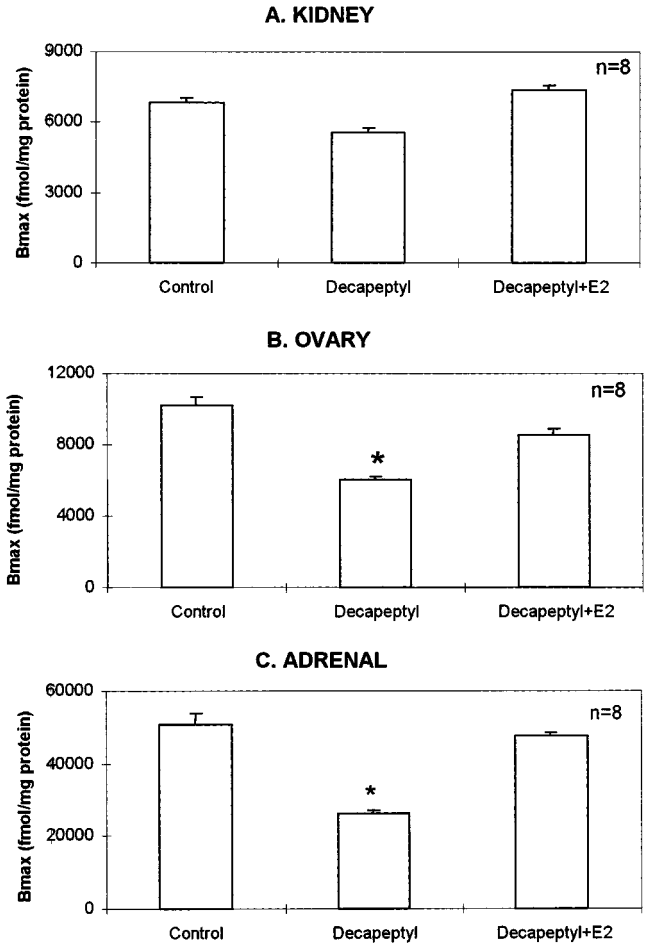

FIGURE 3: [^{3}H]PK 11195 maximal binding capacity in the three tissues following pharmacological castration ($n = 8$ in each group). Results are mean ± SE of PBR densities. *$P < 0.05$ versus control and decapeptyl/E_2-treated rats.

TBS-T solution (20 mM Tris-HCl, pH 7.6, and 14 mM NaCl containing 0.1% Tween 20). After several washes, the membranes were incubated with a primary antibody [either polyclonal anti-18-kDa IBP antibody prepared in our laboratory or the commercial monoclonal anti-32-kDa VDAC (Calbiochem, San Diego, CA) or monoclonal anti-phosphoserine/phosphotyrosine (Sigma)] in 1% dried milk in TBS-T for 2 h. The membranes were washed in TBS-T, followed by 1 h incubation with the appropriate secondary antibody [either anti-rabbit IgG or anti-mouse IgG, both being horseradish peroxidase-linked (Amersham)]. After three washes with TBS-T, the membranes were incubated for 1 min with ECL detection reagent (Amersham) and exposed to Hyperfilm (Amersham) for 30−60 s.

Stripping and Reprobing. Nitrocellulose membranes used for Western blotting were soaked in stripping buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.7, and 100 mM β-mercaptoethanol) with shaking for 30 min at 60 °C and washed twice with TBS-T for 10 min each time. These membranes were used for Western blotting with other antibodies.

Immunoprecipitation. Mitochondrial fractions (50−100 μg of protein) were diluted 1:10 with TBS and incubated with 3.6 μg of the antiporin (VDAC) monoclonal antibody (HPORIN1, Calbiochem) at 4 °C overnight. Immune complexes were then precipitated at 4 °C for 1 h by the addition of 50 μL of protein G (100 μg) coupled to Sepharose 4B

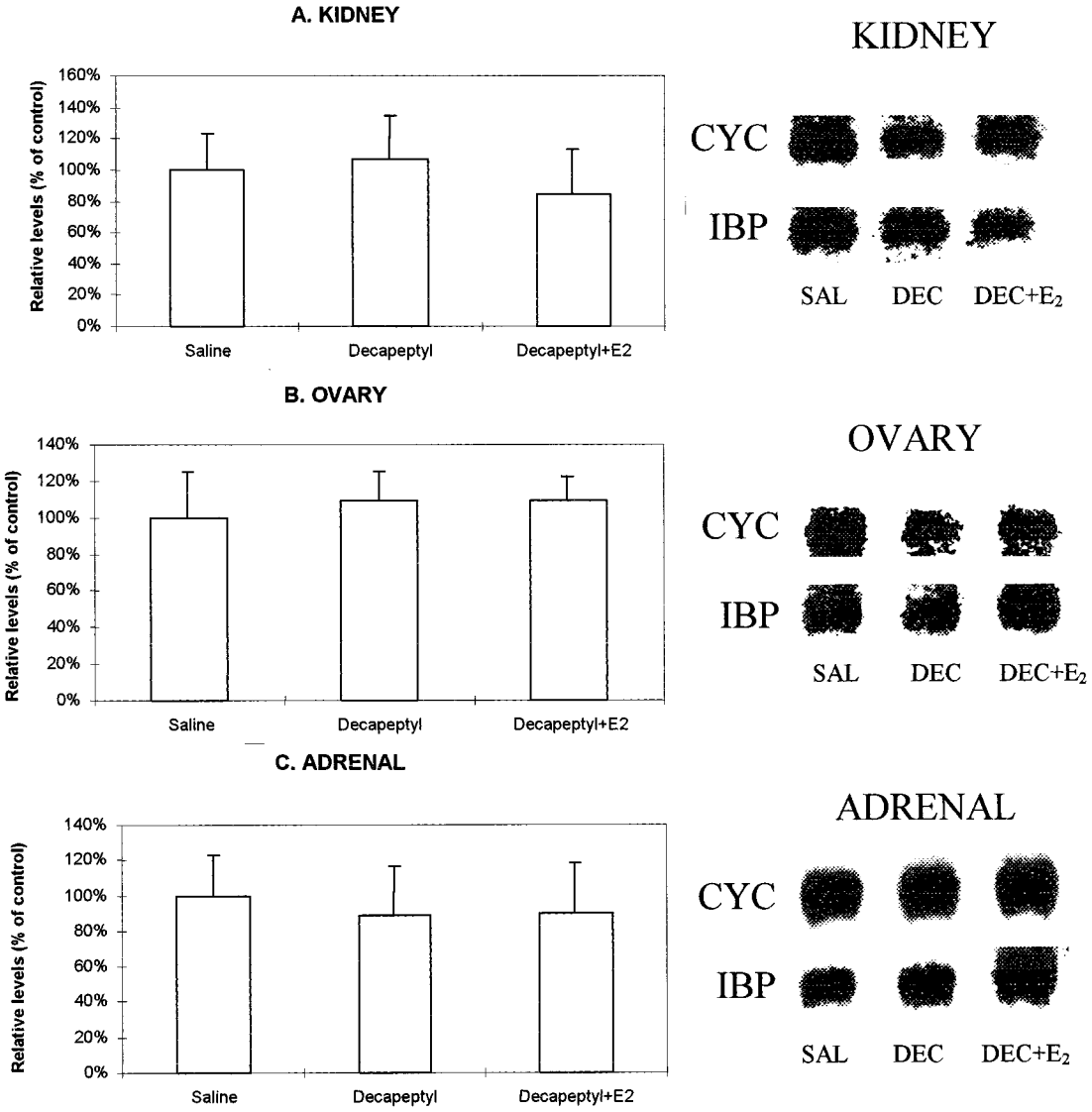

FIGURE 4: IBP subunit steady-state mRNA levels in the decapeptyl-induced ovarian suppression model ($n = 6$ in each group). Analysis of IBP mRNA levels was quantified using a phosphor imager, with normalization to cyclophilin (CYC) mRNA levels. Left panels: histograms summarizing the data for each tissue and treatment. Right panels: representative Northern blot autoradiograms. No significant differences were detected between the tissues and treatments studied.

(Sigma). The beads were washed twice with TBS before dissolving in 2× sample buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% w/v SDS, 0.14 M β-mercaptoethanol, and 0.005% bromphenol blue), boiling for 10 min, and loading onto 4−20% SDS−polyacrylamide gradient gels. Transfer procedure to nitrocellulose membranes and Western blotting were performed as described above.

Statistical Analysis. Results are expressed as mean ± SE. One-way ANOVA and Student-Newman-Keuls post hoc analysis as well as Kruskal-Wallis analysis were used as appropriate. Statistical significance was defined as $P < 0.05$.

RESULTS

Efficiency of Ovarian Suppression. Decapeptyl administration resulted in a significant reduction in the weights of ovaries ($F = 12.209$, $df = 23$, $P < 0.001$) and adrenals ($F = 16.280$, $df = 23$, $P < 0.001$) but not of the kidney (Figure 1). As shown in Figure 2, chronic decapeptyl treatment was associated with persistent suppression of serum progesterone levels. (Kruskal-Wallis value 17.279, $P < 0.001$). These results validate the efficacy of the suppressive effect of decapeptyl on ovarian activity (castrating-like effect) (*27*, *28*).

PBR Binding Characteristics. Decapeptyl treatment was associated with significant (40 and 48%) reduction in [^{3}H]-PK 11195 maximal binding capacity in the ovaries ($F = 26.5$, $df = 18$, $P < 0.001$) and adrenals ($F = 128.14$, $df = 15$, $P < 0.001$), respectively, but not in the kidney. This effect was prevented by co-administration of E_2 (Figure 3). The down-regulatory effect of decapeptyl treatment on PBR density was not accompanied by a significant alteration in the affinity of the ligand to the receptor (kidney, 1.15−2.58 nM; ovary, 0.94−1.78 nM; and adrenal, 2.48−4.95 nM). In an attempt to determine the biochemical level at which the in vivo ovarian suppression affected PBR binding capacity, we initiated experiments to examine PBR regulation at the transcriptional, posttranscriptional, translational, and post-translational levels.

mRNA Regulation. At the transcriptional level, we attempted to measure ovarian suppression-induced changes in

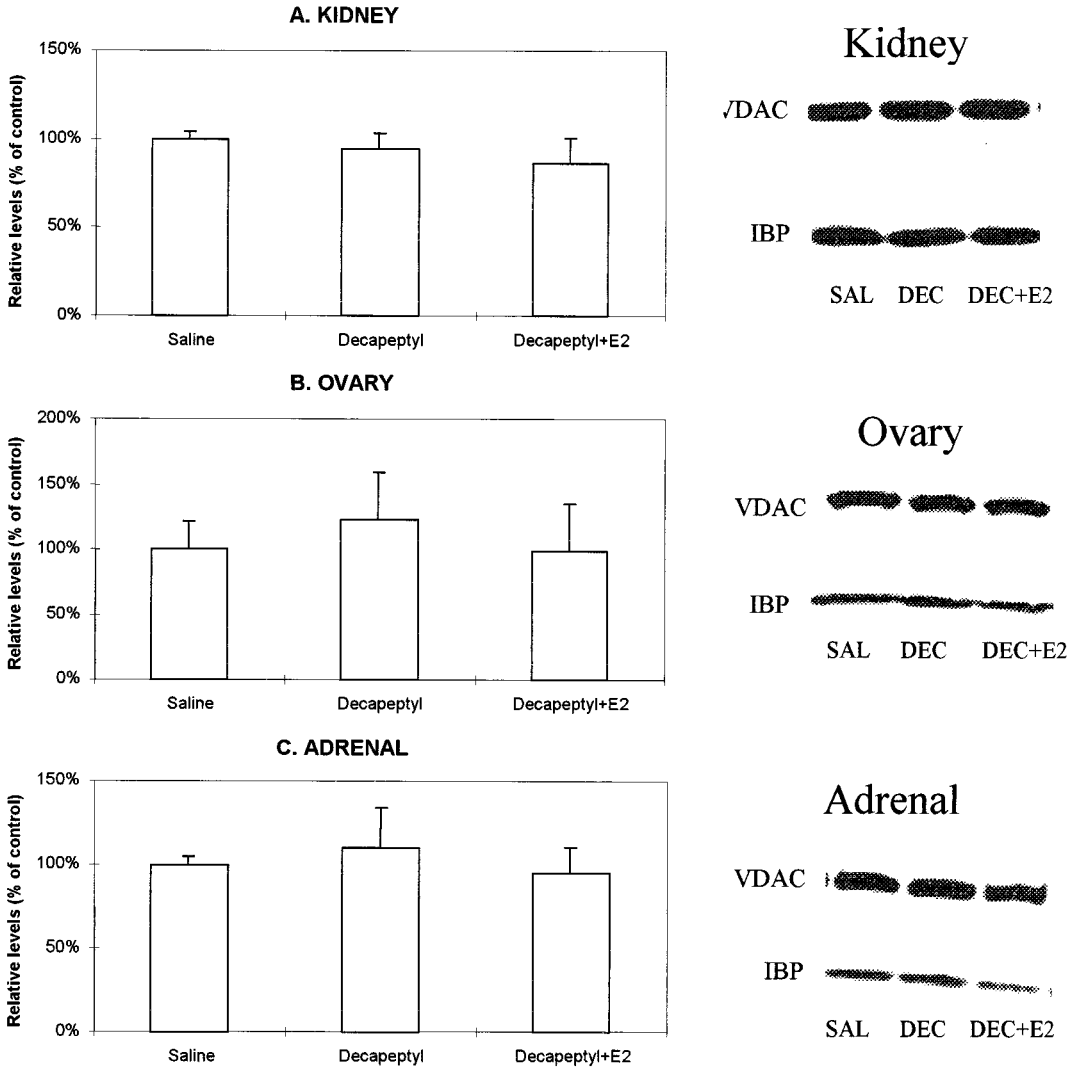

FIGURE 5: Western blot analysis of the 18-kDa IBP subunit following decapeptyl administration ($n = 6$ in each group). Mitochondrial proteins (10−40 μg) were loaded onto gradient SDS gels and transferred to nitrocellulose. Blots were probed by Western analysis with antibodies against 18- and 32-kDa subunits. Left panels: Histograms summarizing Western blot analysis data for the 18-kDa IBP subunit. Right panels: representative examples of such primary data together with those of VDAC. No significant differences between treatments were detected in IBP and VDAC protein levels.

steady-state IBP mRNA levels as well de novo IBP RNA run-on transcription rates. As shown in Figure 4, Northern analysis did not reveal any change in the IBP steady-state mRNA levels (following normalization to cyclophilin). A single band of mRNA was visualized at the appropriate molecular size (900 nucleotides) in the two steroidogenic tissues (ovary and adrenal) as well as in the nonsteroidogenic kidney tissue. Furthermore, as there was only the same single mRNA band in the basal and treatment groups, this was inconsistent with splicing as a mode of IBP regulation in this case. Additionally, pilot de novo transcriptional analysis (run-on assay) of the IBP genetic locus also gave no indication for changes in regulation of de novo RNA synthesis (data not shown).

Protein Regulation. At the level of protein regulation, we measured changes of the 18-kDa IBP and the 32-kDa VDAC subunits of PBR separately, using Western analysis. Antibodies were prepared in our laboratory to a peptide corresponding to the exon-4 domain of the rat IBP gene. In addition, a commercial antibody to the VDAC subunit (HPORIN1) was also used to assess the involvement of the VDAC subunit in the down-regulation of PBR ligand binding. We found no changes in the levels of the 18-kDa IBP or the 32-kDa VDAC PBR subunits when comparing the different treatments in the three tissues studied (Figure 5). Furthermore, to determine whether differences in the phosphorylation state of both the 18-kDa IBP and the 32-kDa VDAC subunits of PBR contributed to changes in PBR binding capacity, immunoprecipitation/Western analysis was undertaken. No changes between the treatment groups for the phosphotyrosine and phosphoserine levels for both of these two PBR subunits in the three studied tissues were observed (see a representative example of such data for the adrenal in Figure 6 that is typical of all three tissues).

Posttranslational Regulation. At this level, it is possible that the interaction between the different PBR subunits may be critical to the binding capacity of the whole PBR complex. To address this issue, we used a combined immunoprecipitation/Western analysis approach, focusing on the VDAC and IBP PBR subunits. In this experiment, a commercial antibody to human porin type I (HVDAC1) was used to immunoprecipitate the PBR complex for each tissue. The resultant precipitate was then resolved by PAGE, transferred to nitrocellulose, and then probed with our anti-18-kDa IBP

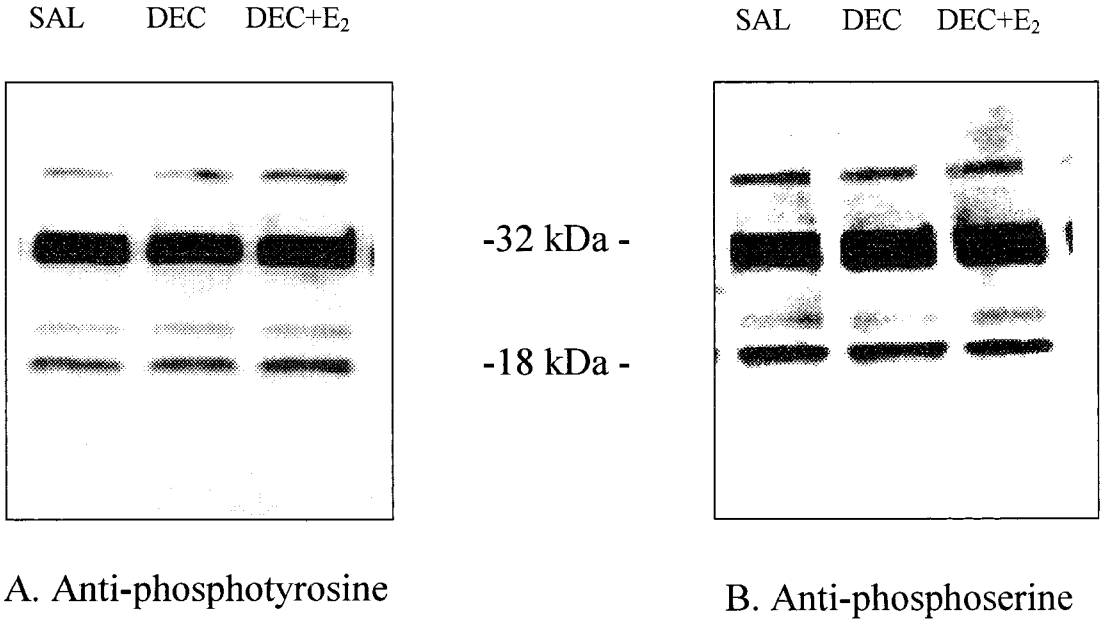

FIGURE 6: Phosphorylation state of adrenal PBR subunits IBP and VDAC ($n = 3$ for each group). PBR complex was immunoprecipitated using anti-VDAC antibody, and the resultant precipitated complex was loaded onto denaturing SDS gels. Blots were used for Western analysis using anti-phosphoserine and anti-phosphotyrosine monoclonal antibodies. No differences in the phosphorylation states were noticed between treatments.

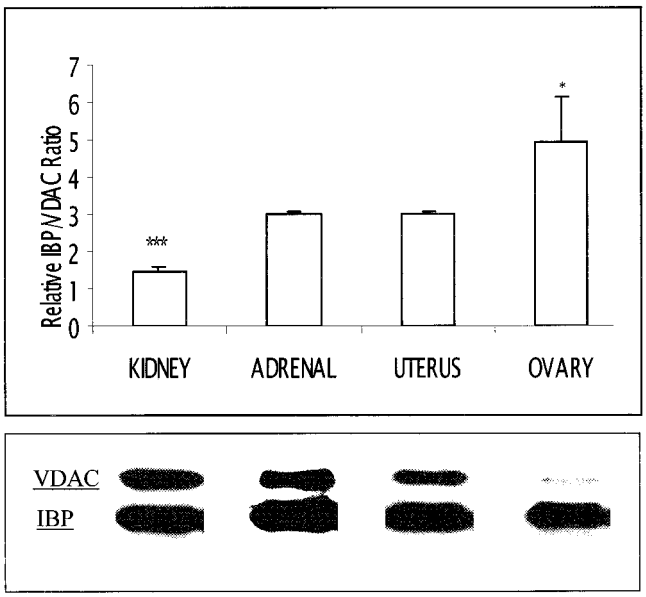

FIGURE 7: Tissue-specific relative ratios of PBR subunits in untreated rats. The PBR complex was immunoprecipitated using an anti-VDAC antibody. The resultant precipitated complex was subjected to PAGE followed by Western transfer to nitrocellulose, and the resultant membranes were then probed simultaneously with both 18- and 32-kDa subunit antibodies. The resultant relative ratios represent the actual molar relationship between PBR subunits, due to the optimized double staining used in this experiment. *$P <$ 0.05 versus adrenal and uterus ($n \geq 8$ for each tissue); ***$P <$ 0.001 versus all other columns.

polyclonal antibody as well as the monoclonal anti-32-kDa VDAC antibody. To confirm resultant ratios, similar experiments were also undertaken that reversed the order of the antibodies. These experiments clearly showed that upon the immunoprecipitation of either VDAC or IBP, the complementary subunit was also precipitated (Figures 7 and 8). In untreated rats ($n \geq 8$), the IBP/VDAC ratio was found to be tissue specific (Figure 7). Specifically, we found that there was 1.5-fold IBP per VDAC in the kidney, while in both adrenal and uterus there was twice as much IBP per VDAC. Additionally, in the ovary there were five IBP subunits for each VDAC subunit of PBR (Figure 7).

To determine whether decapeptyl treatment could affect the IBP/VDAC ratio, we examined mitochondrial membrane protein extracts from treated and control rats. Again, we found that indeed the 18-kDa IBP PBR subunit was coprecipitated with the VDAC protein, which was direct evidence for physical contact between the IBP and VDAC subunits. Following decapeptyl administration, the ratio between IBP and VDAC subunits changed in the adrenal; that is, for the same amount of eluted IBP, there was less VDAC present compared with the saline control (Figure 8). This could also be understood as illustrated in Figure 9. Even though a trend toward a change in subunit interaction was also found for the ovary, no such change was found in the kidney.

E_2 treatment did not prevent these changes in PBR subunit interactions in either adrenal or ovary.

DISCUSSION

While certain sequence characteristics as well as its conservation through evolution suggest that the PBR may be a housekeeping gene, its main physiological role is still an enigma (*27*). Accumulating data indicate the involvement of PBR in steroid production (*36*−*40*). These data include the effects of hormones on PBR density levels, as well as the regulation of steroidogenesis by PBR ligands. For example, hypophysectomy causes a dramatic decrease in PBR density in the adrenal gland and testis, which is reversed upon appropriate trophic hormone treatment (*2*, *22*, *28*). Other studies have shown that diazepam-binding inhibitor (DBI), a potential endogenous ligand for PBR, regulates steroidogenesis, activated by adrenocorticotropic hormone (ACTH) and luteinizing hormone (LH) via DBI binding to PBR (*20*, *29*−*31*). These findings, together with the observation that PBR are present at high densities in steroidogenic

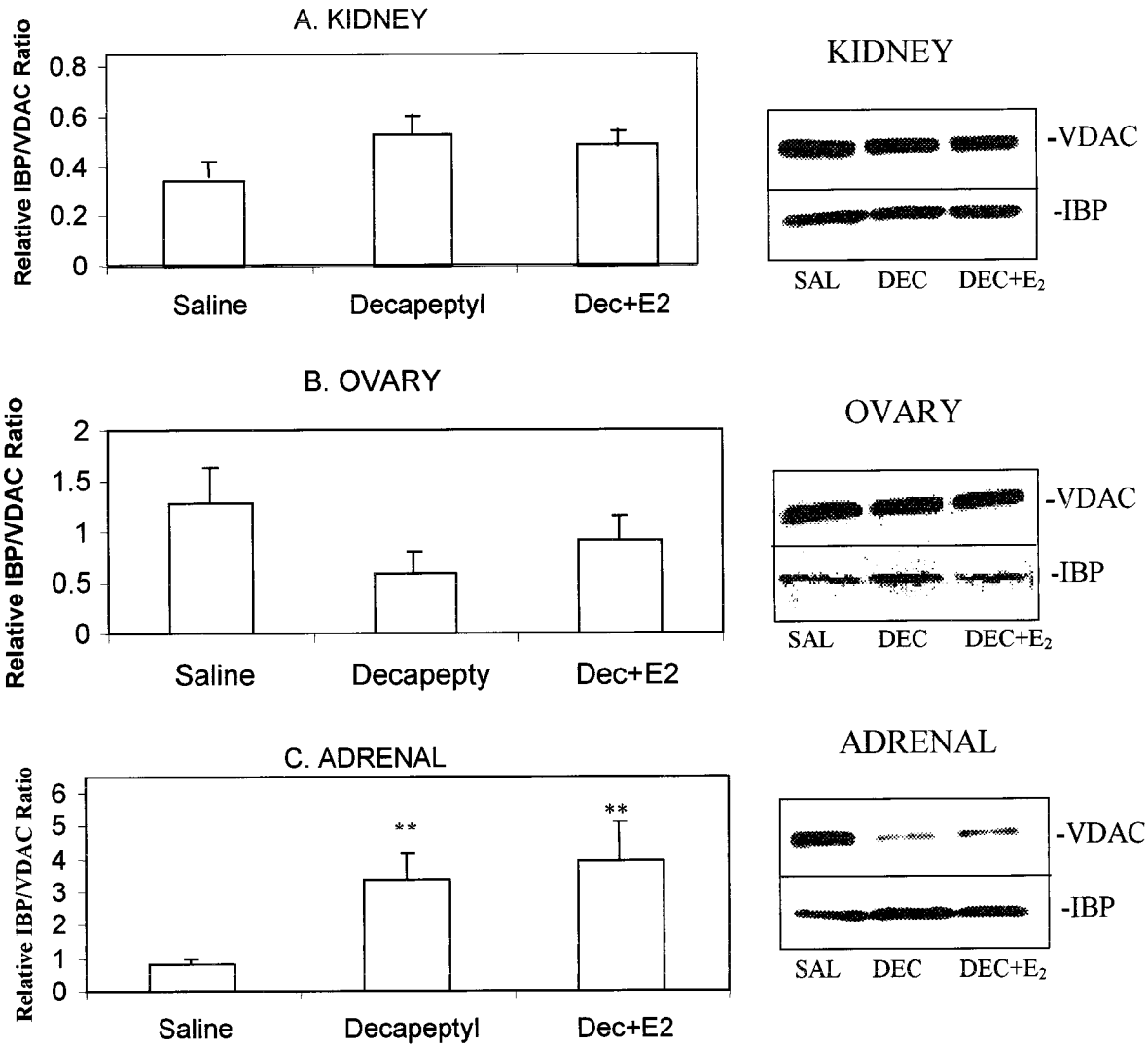

FIGURE 8: Posttreatment alterations in relative ratios of PBR subunits 32-kDa VDAC/18-kDa IBP ($n = 4$ for each group). PBR complex was immunoprecipitated using an anti-VDAC antibody. The resultant precipitated complex was loaded onto denaturing SDS gels and transferred to nitrocellulose. The blots were used for Western analysis with separate antibodies against the 18- and 32-kDa subunits. These resultant relative ratios represent the trend differences between the PBR subunit ratios following different treatments, due to the separate antibody staining used in this experiment. $^{**}P < 0.01$ versus control rats.

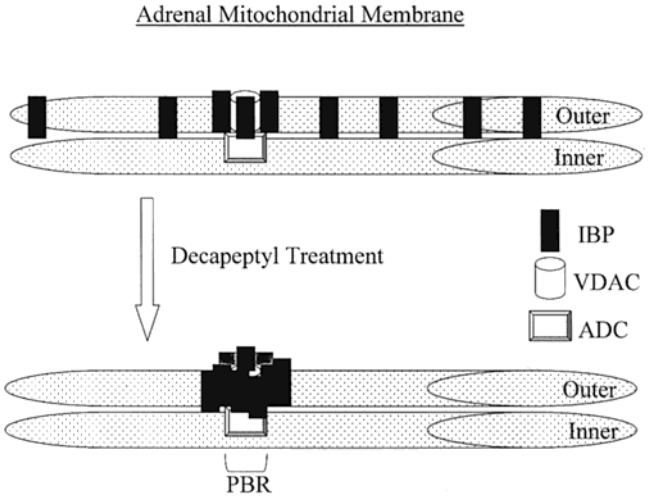

FIGURE 9: Schematic illustration of the effect of decapeptyl treatment on rat adrenal mitochondrial PBR subunit interactions.

organs (*3*, *5*) and are located mainly on the outer mitochondrial membrane (*13*−*15*), further reinforce the notion that PBR play a major role in steroid biosynthesis.

Steroid sex hormones are under the control of gonadotropins in the male and female reproductive system. The regulation of biosynthesis and secretion of the gonadotropins, i.e., LH and follicle-stimulating hormone (FSH), is critical for normal reproductive function. The synthesis and release of these two pituitary glycoproteins is controlled by a complex interaction of multiple factors, including GnRH, a hypothalamic decapeptide. GnRH is released into the hypophyseal portal circulation and transported to the anterior pituitary in a pulsatile manner, where it binds to specific high-affinity receptors and serves as the major regulator of LH and FSH secretion. However, chronic treatment with highly potent and long-acting analogues of GnRH, such as decapeptyl, has been shown to down-regulate GnRH receptors and reduce pituitary gland response to GnRH, thereby causing decreased secretion of gonadotropin and gonadal sex steroids (*41*, *42*). Chronic GnRH analogue administration is one of the clinical approaches intended to interfere with sex hormone production without surgical removal of the gonads.

Chronic administration of decapeptyl has been shown to suppress the hypothalamic−pituitary−ovarian axis (*41*, *42*).

Such an effect was also obtained in our study, as revealed by the reduction in ovarian weight and ovarian PBR density. Interestingly, decapeptyl administration had a large impact on adrenal gland PBR binding capacity. Some studies have suggested an interaction between the hypothalamic−pituitary−ovarian axis and adrenal activity. Administration of GnRH has been shown to stimulate ACTH release in men and women with ACTH-producing adenomas (*43*, *44*) as well as in ovariectomized rat anterior hemipituitaries, perfused in vitro (*45*). It has also been shown that adrenal androgen synthesis in women is at least partially under ovarian control (*46*). To the best of our knowledge, there are no data about an inhibitory effect of chronic GnRH treatment on adrenal function in rats.

Our data indicate that decapeptyl-induced suppression of pituitary gonadotropin release was associated with a reduction in PBR binding capacity in the female steroidogenic tissues (ovary and adrenal), but not in total PBR subunit protein content. This PBR depletion was not prevented by E_2 replacement treatment. The alterations observed in the maximal binding capacity for the ligand [^{3}H]PK 11195 in E_2-treated rats might be attributed to the presence of an excess concentration of E_2. However, this possibility seems unlikely, since in vitro experiments demonstrated that E_2 concentration up to 0.1 μM did not affect [^{3}H]PK 11195 binding to PBR (*47*). Furthermore, the procedure used for membrane preparation removes most of the estradiol, which could be present in the homogenate. Thus, it seems that the changes in [^{3}H]PK11195 binding imply a modulatory effect of chronic E_2 treatment on PBR and not in vitro interference with binding capacity at the membrane level. We had hypothesized that this significant reduction in PBR binding capacity was probably due to alterations in one or more of the various levels of gene expression, specifically transcription, posttranscription, translation, and posttranslation. In this study, we examined these levels of PBR gene expression, starting with steady-state IBP mRNA levels as well as de novo IBP RNA run-on transcription. This was followed by Western blot analysis of the 18-kDa IBP and the 32-kDa VDAC protein content. Moreover, we attempted to measure the phosphorylation state of these two PBR subunits, as well as any physical interaction between the same two subunits. Interestingly, we found no changes in the transcription, posttranscription, translation, and phosphorylation of the IBP and VDAC protein subunits when comparing the organs studied in decapeptyl-treated and control rats. In contrast to this, we did find changes in IBP and VDAC PBR subunit interactions, which may be involved with or at least coincident with the reduction in PBR binding capacity in these steroidogenic tissues following decapeptyl treatment. As illustrated in Figure 9, we understand that decapeptyl treatment induced an increase in the number of IBP molecules binding to each VDAC molecule within the mitochondrial pore. The consequential blocking of this mitochondrial pore resulted in reduced PBR ligand binding. Such changes were not found in the nonsteroidogenic renal tissue.

Examination of MA-10 Leydig cell mitochondrial preparations by transmission electron and atomic force microscopic procedures (*48*) indicated that the 18-kDa IBP PBR subunit is organized in clusters of four to six molecules with an interrelationship to favor the formation of a single mitochondrial pore. Previous observations indicated that the 18-kDa IBP is structurally (*8*) and functionally (*49*) associated with the 32-kDa VDAC protein. McEnery et al. (*8*) demonstrated, by means of solubilization, photoaffinity labeling, and immunological detection of the PBR complex, that the PBR is comprised of three distinct protein subunits (18, 30, and 32 kDa). Garnier et al. (*49*) showed that the PBR is a multimeric complex in which the benzodiazepine binding site requires both the 18-kDa IBP and 32-kDa VDAC subunits. The VDAC subunit is an ion channel that is located in the outer mitochondrial membrane and in the junctions between the outer and inner membranes (contact sites), where it may complex with the ADC (*50*). The VDAC subunit is assumed to allow transport of metabolites and small molecules between the cytoplasm and the inner mitochondrial membrane (*50*, *51*). Furthermore, other investigators have suggested that this same mitochondrial pore, composed of VDAC, ADC, and IBP, plays an essential role in the apoptotic process (*41*). Taking into account these observations, together with our findings of direct physical contact between the 18-kDa IBP and 32-kDa VDAC, it seems that IBP/VDAC subunits form a putative pore in the outer mitochondrial membrane, allowing small molecules of molecular mass < 6 kDa, including cholesterol (*51*), to translocate into the inner mitochondrial membrane. On the basis of our data, and the apoptotic model described by Green and Reed (*52*), we would like to suggest that this mitochondrial pore has an active as well as an inactive conformation. A theoretical three-dimensional model of the PBR was developed using molecular dynamics (*53*). This model suggested that five α-helices cross one phospholipid bilayer of the outer mitochondrial membrane and can serve as a cholesterol carrier. It was shown that PBR could accommodate a cholesterol molecule and function as a channel (*53*).

Although the 18-kDa IBP subunit was able to bind PK 11195 at micromolar levels in vitro in the absence of VDAC and ADC (*54*), it is possible that interactions between the PBR subunits are necessary for optimal PBR ligand binding (nanomolar affinity) in the rat in vivo. Furthermore, PBR subunit interactions are necessary for the formation of the putative active mitochondrial pore in the outer mitochondrial membrane, allowing small molecules, including cholesterol (*51*), to translocate into the inner mitochondrial membrane.

E_2 is the dominant ovarian steroid because of its biological potency and diverse physiological effects on peripheral target tissues (*55*). For this reason, many hormone deficiency models in the rat use E_2 as a replacement treatment (*28*, *56*, *57*). In our study, the reduction in PBR binding densities observed in the ovarian and adrenal steroidogenic tissues was prevented by E_2 co-administration; however, this E_2 treatment did not affect the alteration in the IBP/VDAC ratio observed in decapeptyl-treated rats. It is possible that combined E_2/progesterone treatment is necessary for complete restoration of the hormonal balance, as demonstrated in another system (*58*) and would be an interesting issue to be further investigated. Thus, it could be that the combined hormone treatment would lead to the normalization of the IBP/VDAC ratio. It is also possible that other proteins, e.g., StAR and Ca^{2+} channel components, may influence the assembly of the PBR subunits and the formation of an active mitochondrial pore. The ADC has also been shown to be a part of the PBR complex (*8*, *52*). One of its roles is to transport adenine

nucleotides across the mitochondrial membrane. Although in the current study we did not measure possible alterations in ADC expression, the role of ADC in PBR subunit interactions cannot be disregarded within the extent of the mitochondrial pore (*52*).

A classic example of subunit interaction regulation of proteins is the G-protein family (*59*). G-proteins consist of three subunits: α, β, and γ. In the resting state, the G-proteins exist as an unattached $\alpha\beta\gamma$ trimer, with GDP occupying the site on the α subunit. When a receptor is occupied by an agonist molecule, a conformational change occurs, causing GDP/GTP exchange. As a result, the α-GTP is dissociated from the $\beta\gamma$ subunits, becoming the active form of the G-protein. The process is terminated when GTP hydrolyzes to GDP and the α-GDP reassociates with $\beta\gamma$. This is an example of subunit interaction in which the dissociation and reassociation of the three subunits are necessary for normal function of the G-proteins. In our study, we saw a different example of subunit interaction, in which the possible association and stoichiometry (subunit ratio) of at least two of the PBR subunits (IBP and VDAC) may be involved in the regulation of PBR binding capacity. However, since the E_2 treatment restored the PBR binding capacity to control values without normalizing the IBP/VDAC ratio, we assume that other factors or mechanisms are also necessary. It is possible that other E_2-dependent but mitochondrial pore-independent mechanisms play a role in the maintenance of PBR binding capacity in steroidogenic tissues.

In conclusion, the current study not only demonstrates a role for changes in PBR subunit interactions in the regulation of PBR receptor action, but also reinforces earlier studies suggesting the existence of a mitochondrial pore. This mitochondrial pore, in addition to its function as a cholesterol transporter as well as a regulator of programmed cell death (*52*), may also be the gateway to other transport systems, such as the TIM/TOM mitochondrial protein transport system (*60*).

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