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# A specific membrane binding protein for progesterone in rat brain: Sex differences and induction by estrogen

(conjugated progesterone/hypothalamus/corpus striatum/affinity chromatography)

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ABSTRACT **Progesterone conjugated to bovine serum albumin (BSA) was used as a probe to study sex differences and the effects of hormonal status on binding of progesterone to crude synaptosomal membrane preparations ($P_2$) derived from the mediobasal hypothalamic–anterior hypothalamic–preoptic area or the corpus striatum. Binding of $^{125}$I-labeled BSA linked to progesterone at the 11 position of the steroid (P-11-BSA) was decreased by competition with unlabeled P-11-BSA or P-3-BSA (in which progesterone is bound to BSA at the 3 position). P-3-BSA displayed higher affinity than P-11-BSA. Hypothalamic and striatal preparations from adult females show high specific binding (60–80%) to the progesterone-BSA conjugate. Specific binding was reduced more than 80% 14 days after ovariectomy. Estrogen treatment (10 μg per rat for 4 days) of 14-day ovariectomized rats restored specific binding to levels equivalent to intact females. In contrast, adult males displayed drastically reduced or no specific binding in either tissue. No specific binding was detected after orchidectomy. Estrogen treatment of orchidectomized animals induced specific binding sites similar to those in intact females. Additionally, an affinity probe was developed by linking primary amines on the P-3-BSA conjugate to agarose activated aldehydes in an AminoLink column. A digitoxin-solubilized fraction from female rat $P_2$ cerebellum preparations yielded a single major band after affinity purification with an estimated molecular mass of 40–50 kDa in an SDS/PAGE system after silver stain. These results show a reversible sex difference in the specific binding of progesterone to synaptosomal membrane sites in the central nervous system of male and female rats which is dependent on estrogen.**

Progesterone (P) is known to influence a variety of physiological functions in the central nervous system (CNS) (1). The effects of P on the luteinizing hormone-releasing hormone (LHRH) pulse generator (2) and female sexual behavior (3, 4) are well documented. Sex differences in the neuroendocrine control of gonadotropin secretion may be attributed to sex steroid-regulated functions of the ventromedial nuclei and the preoptic area (POA) (for review see ref. 5). The mechanism for these actions requires activation of the genome by interaction with nuclear receptors and ensuing alteration of gene expression (for review see ref. 6).

Considerably less attention has been given to the rapid neurotropic actions of steroids (onset in milliseconds to minutes) which include, but may not be limited to, alterations in neuronal excitability and modification of specific neurotransmitter receptors and their effectors (7, 8).

For example, P results in rapid release of LHRH from hypothalamic tissues (2) and enhances amphetamine-stimulated dopamine release (9). P infusion into the cerebellum decreases electrophysiological responsiveness to glutamate while potentiating the effect of γ-aminobutyric acid (10). P administration to the ventromedial nuclei causes increases in the number and area covered by oxytocin receptors within 30 min (11). The time course suggests a nongenomic mechanism mediated by putative membrane receptors. Additional evidence supports the presence of membrane binding sites for P (2, 11–14) and corticosteroid (15) in neuronal tissues. P also binds σ receptors in the guinea pig brain (16).

Ke and Ramirez (14) developed an approach to examine nongenomic actions of P by using conjugated or immobilized steroids. P is coupled at the 11 or 3 position, by means of a carbodiimide reagent, to a large protein, bovine serum albumin (BSA) (17). Biological activity has been demonstrated by using P-BSA conjugate (18–22).

This study examines several aspects of the interaction of P with a putative neuronal membrane binding protein in the mediobasal hypothalamic–anterior hypothalamic–preoptic area (MBH–AHA–POA) and the corpus striatum (CS). Additionally, we sought to purify from the cerebellum a soluble membrane protein with affinity for P.

## MATERIALS AND METHODS

**Animals.** Adult male and female Sprague–Dawley rats (80–110 days old) were used. Animals were maintained on a 14:10 hr light/dark cycle (lights on at 0700) with food and water available ad lib. Where hormonal manipulations were necessary gonadectomy was performed while the animals were under ether anesthesia. Estradiol benzoate ($E_B$; 10 μg/0.1 ml of sesame oil) was administered by daily subcutaneous injection at 1000 on each of 4 consecutive days, beginning 14 days after gonadectomy. Although this dose is rather high, it was chosen because it has previously been shown to induce specific biological effects of estrogen (23). Control animals received injections of sesame oil vehicle. Tissue samples were obtained from eight groups of animals: I, intact female; II, 7-day ovariectomized (OVX) female; III, 14-day OVX female; IV, 14-day OVX + oil-injected female; V, 14-day OVX + $E_B$-injected female; VI, intact male; VII, 21-day orchidectomized + oil-injected male; and VIII, 21-day orchidectomized + $E_B$-injected male.

**Preparation of Crude Membrane Pellets ($P_2$ Fraction).** Animals were sacrificed by decapitation between 0900 and 1000, and appropriate brain regions were removed within minutes. After the top of the skull had been removed the brain was exposed and the following structures were removed with a small curved surgical scissor. First, the cerebellum was removed from the brainstem by cutting the cerebellar peduncle. Second, the two cerebral hemispheres were separated and the cortical tissue overlying the CS was blunt dissected to expose the entire CS, which was then easily removed from

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Abbreviations: P, progesterone; $E_B$, estradiol benzoate; CNS, central nervous system; LHRH, luteinizing hormone-releasing hormone (luliberin); POA, preoptic area; BSA, bovine serum albumin; MBH–AHA, mediobasal hypothalamic–anterior hypothalamic area; CS, corpus striatum; OVX, ovariectomized.

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the surrounding tissue. Last, the hypothalamus was removed by vertical cuts made at the level of the mammillary body, following the hypothalamic sulci and about 1 mm anterior to the optic chiasm. The entire fragment was lifted up with the scissors after making a horizontal cut of about 1–2 mm in depth. Each brain region was placed in a beaker containing ice-cold Tris buffer. Tissues were homogenized in a Teflon glass homogenizer (type C; Thomas) in 20 vol of ice-cold Tris buffer (50 mM Tris·HCl/120 mM NaCl/5 mM KCl/1 mM $MgSO_4$/1 mM $CaCl_2$, pH 7.4 at 4°C). Homogenates were centrifuged at 1000 × *g* for 10 min. Supernatants were centrifuged at 17,000 × *g* for 20 min to obtain $P_2$ pellets. $P_2$ pellets were resuspended in Tris buffer, assayed for protein by the Bradford (24) method, and stored at −20°C for up to 6 weeks. Since the estimated high-affinity binding sites for P-3-BSA in the $P_2$, axolemma, and myelin fractions were 2.7, 3.7, and 0.3 pmol/mg of protein, respectively (14), the binding of P-BSA to $P_2$ membranes represents mainly interactions of P-BSA with the plasma membrane.

**Radioiodination of P-11-BSA.** P-11-BSA was radioiodinated according to the method of Fracker and Speck (25) with slight modifications. A 50-μl portion of Iodo-Gen at 0.04 mg/ml in methylene chloride was dried under reduced pressure at room temperature. Twenty micrograms of 11α-hydroxyprogesterone 11-hemisuccinate conjugated to BSA (P-11-BSA; Sigma catalog no. H-4508) dissolved in 5 mM Tris·HCl at pH 7.4 and 250 μCi (1 Ci = 37 GBq) of $Na^{125}I$ was added to the Iodo-Gen-coated vial. P-11-BSA was chosen as the radiolabeled ligand because it binds specifically to membranes with less nonspecific binding to filters than P-3-BSA. Although P-11-BSA has reduced affinity and limited biological activity (26), it may be considered similar to pharmacological analogs commonly used in receptor binding studies. Total reaction volume was 500 μl. The reaction mixture was applied to a Sephadex G-100 column (1 × 50 cm) to separate bound protein from free radioiodine. The specific activity was calculated from measured radioactivity of the P-11-$^{125}$I-BSA fraction. The ligand was stored at −70°C and was active for up to 8 weeks.

**Radioligand Binding Assays.** The P-11-$^{125}$I-BSA radioligand binding assay has been described elsewhere (14) and updated recently (27). Briefly, the reaction was performed in Tris buffer as above to which 0.08% BSA was added (binding buffer). P-11-$^{125}$I-BSA (≈875 Ci/mmol) was diluted to about 40,000 cpm (≈ 73 pmol) per assay tube. The competing ligand and $P_2$ fraction (12–400 μg of protein) were added. After 30 min [when equilibrium is reached (14)] the reaction was terminated by adding 5 ml of reaction buffer and rapidly filtering under reduced pressure through Whatman GF/C glass fiber filters. Filters were washed by repeating the filtration step with another 5 ml of reaction buffer. The total time for filtration and washing was less than 10 sec. The radioactivity of bound ligand retained on the filter was determined by using a γ counter at 70% efficiency (1185 series; Searle). Ke and Ramirez (14) previously published that binding reaches equilibrium by 30 min.

The above assay was modified for studies involving proteins after solubilization and lyophilized products after affinity chromatography. Fifty microliters (20 nmol) of [$^3$H]P (130 Ci/mmol; Amersham) was added to a microtube. Ten micrograms of solubilized protein or ≈50 ng of protein after column elution was added. Total reaction volume was 100 μl. The reaction proceeded as described above. Filters were collected, and [$^3$H]P was quantitated by using a Beckman LS-3100 liquid scintillation counter (40% efficiency).

**Solubilization and Purification of the Membrane P-Binding Protein.** $P_2$ fractions from adult female cerebellum, previously shown to bind the radioligand (14), were used as a source to purify the putative membrane P-binding protein because of the large amount of tissue available per rat and the fact that after ovariectomy specific binding is no longer detected in these fractions (unpublished results). Moreover, the cerebellum of immature female rats has less specific binding before puberty than after puberty (27). $P_1$ membranes from the cerebellum served as a control because this fraction, which contains nuclei, unbroken cells, and other cellular debris, does not bind the radioactive probe (14). The solubilization procedure was based on that of Caron and Lefkowitz (28). Frozen membranes were thawed and washed in 5 vol of 25 mM Tris·HCl/2 mM $MgCl_2$/1 mM EGTA/10 mM NaF, pH 7.4. Following centrifugation at 17,000 × *g* for 30 min, the pellets were solubilized in 1 ml of binding assay buffer containing 1% digitonin (Sigma). After standing on ice for 30 min with periodic agitation, the suspension was centrifuged at 125,000 × *g* for 120 min. The soluble fraction (containing the binding protein as determined by binding assay; see Fig. 6) was pooled to a protein concentration of 2 mg/ml and applied to an affinity chromatography column.

**Affinity Chromatography.** Reductive amination was performed to link primary amine groups on the P-3-BSA complex to agarose activated aldehydes in an AminoLink column (Pierce). Coupling efficiency was over 80%. The solubilized cerebellar $P_1$ (control) or $P_2$ fractions were applied to the column and incubated overnight at 4°C. The column was washed with Tris binding buffer to remove unbound protein. The bound fraction was eluted with 15 ml of 0.1 M acetic acid. One-milliliter fractions were collected and assayed for protein by $A_{280}$. Appropriate fractions were lyophilized to concentrate protein prior to one-dimensional SDS/PAGE.

**SDS/PAGE.** After resuspension of lyophilized protein (before or after affinity chromatography) in 30 μl of double-distilled water, an 8-μl sample was applied to a 4–20% polyacrylamide gradient gel. SDS/PAGE was performed under nonreducing conditions (230 mV for 40 min). Gels were stained with Coomassie blue or silver stain (Gelcode Silver Stain; Pierce).

**Data Analysis.** Displacement curves were plotted and the $K_d$ for P-11-BSA was calculated with the nonlinear curve fitting LIGAND program (29). $K_i$ for competing ligands was calculated by using the Cheng–Prusoff equation (30). Differences between groups were analyzed by one-way analysis of variance (ANOVA). $P \leq 0.05$ was considered significant.

## RESULTS

**Comparison of Binding Characteristics of P-11-BSA and P-3-BSA.** Fig. 1 shows the results of a competitive displacement study comparing binding characteristics of P-11-BSA and P-3-BSA. P-3-BSA exhibits significantly greater affinity for the P membrane site than P-11-BSA does. The dissociation constant ($K_d$) for P-11-BSA is 1622 ± 466 nM, while the $K_i$ for P-3-BSA is 52 ± 8.3 nM, values within the range of previous published data (14). Competitive inhibition has also been demonstrated by using free P (in 0.0001–10% dimethyl sulfoxide, which does not interfere in the assay) with a $K_i$ of 465 nM and a Hill binding constant of ≈200 nM, indicating that the binding sites for P in the membranes are of low affinity (Fig. 2). On the basis of raw numbers, the affinity of free P appears to be only 10% of the affinity of P-3-BSA. However, one must consider that the $K_d$ for P-11-BSA and the $K_i$ for P-3-BSA were calculated by using the molecular weight of the entire steroid-BSA complex, which has ≈29 mol of P per mol of BSA. When the $K_i$ for P-3-BSA is recalculated using the molecular weight and concentration of only the P portion of the conjugate, the $K_i$ is not significantly different from that of free P. Moreover, the dynamics of interaction between free P and the binding sites are probably different from those of P-3-BSA and the binding sites, since the steroid is liposoluble and the conjugate is hydrophilic.

**Effects of Sex on P-11-$^{125}$I-BSA Binding to $P_2$ Fractions.** Fig. 3 shows a comparison of binding characteristics of P-11-BSA

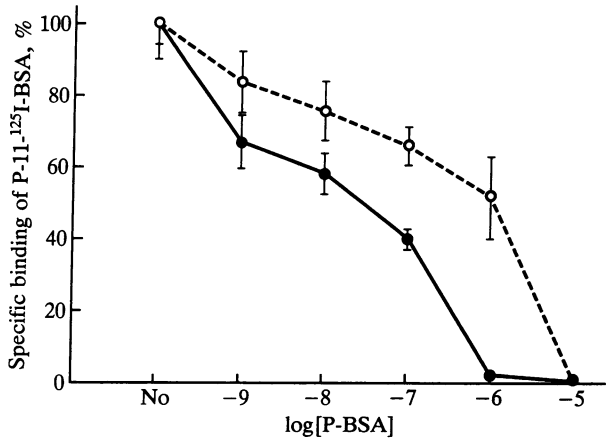


FIG. 1. Competition curve showing specific binding of P-11-$^{125}$I-BSA to $P_2$ fractions prepared from adult female MBH–AHA–POA tissue in the presence of unlabeled P-11-BSA (○) or P-3-BSA (●) at various concentrations. Each tube contained 50 μg of protein. Specific binding was determined by subtracting cpm obtained in the presence of 10 μM unlabeled P-11-BSA or P-3-BSA (P-BSA nonspecific binding). "No" indicates specific binding in the absence of competitor. Data are presented as percent specific binding in the absence of competitor. Specific binding for P-3-BSA was 6443 ± 517 cpm of P-11-$^{125}$I-BSA and specific binding for P-11-BSA was 9994 ± 479 cpm; these values were 60–80% of total binding. Data are presented as mean ± SEM for seven experiments; SEM falls within symbol size where error bars are absent.

in adult male and female rats. P-3-BSA was chosen as the competing ligand because it has been shown to release LHRH (20) and dopamine (21) *in vitro* and has the highest affinity for the membrane binding site. Because the objective of this experiment was to determine sex differences in P-BSA binding and not to examine changes in binding that might occur during the estrous cycle, no attempt was made to discern estrous cycle stage in adult female animals. Intact females show significantly higher specific binding (Table 1) than males. Furthermore, 21 days of castration reduces binding in male rats to levels not significantly above background.

**Effects of Estradiol on Membrane P Binding in Male Rats.** The present data clearly demonstrate (Fig. 4) that a large dose of $E_B$ (10 μg per rat) administered to 21-day castrate animals by subcutaneous injection on each of four consecutive days prior to sacrifice induces P binding at a membrane site in both hypothalamic and striatal tissues (Table 1).

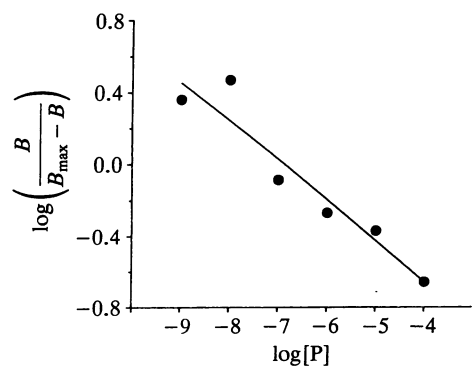


FIG. 2. Hill plot of competition studies using free P dissolved in dimethyl sulfoxide (highest concentration 1%) as the competitor and P-11-$^{125}$I-BSA as the ligand. $B$, cpm bound; $B_{max}$, cpm bound in the absence of competitor. Each tube contained 50 μg of protein of $P_2$ fractions prepared from adult female MBH–AHA–POA tissue. Data correspond to two experiments (each dose was run in duplicate). The mean values for the four determinations are shown. The SEMs were less than 10%. The slope is close to 1.

**Effects of Estradiol on Membrane P Binding in Female Rats.** Both hypothalamic and striatal preparations show highly significant diminution in binding 14 days after ovariectomy ($P < 0.001$) (Fig. 4*A*). Interestingly, no significant reduction in binding was seen in hypothalamic preparations 7 days after ovariectomy, while binding in the striatum was reduced ≈50% from levels observed in the intact condition ($P < 0.05$).

The effect of estradiol replacement (10 μg for 4 days) on P binding to MBH–AHA–POA and CS membrane fractions prepared from 14-day OVX females is shown in Fig. 4*B*. This treatment restored binding characteristics to those exhibited by intact females.

**Affinity Chromatography and SDS/PAGE.** The results of affinity chromatography and SDS/PAGE are shown in Fig. 5. The coupling efficiency for P-3-BSA binding to agarose activated aldehydes in the gel bed was over 80% (data not shown). An example of the amount of protein in homogenates, $P_2$ fractions, and soluble fractions obtained from cerebellum of intact female rats is displayed on the right of Fig. 5*A*. Because previous experimentation had shown high-affinity binding of P-BSA in the cerebellum, with the numbers of binding sites for P-BSA the same as in the hypothalamus and striatum (14), and because a significantly greater amount of tissue can be obtained, the cerebellum was chosen for purification experiments. Starting with cerebellum from four rats, the solubilization procedure yielded 337 μg of protein. Solubilization was repeated to obtain enough tissue to apply 2 mg/ml to the affinity column. $P_1$ fractions were solubilized for use as a control, since such preparations do not bind the P-BSA conjugate (14). Binding of [$^3$H]P to proteins after solubilization was displaceable by 10 μM P (Fig. 6). Binding of P-11-$^{125}$I-BSA to the soluble fraction has also been demonstrated (data not shown). Analysis of such binding data has shown that the soluble fraction contains ≈1.3 pmol of binding protein per mg of protein, with a 25% recovery from the $P_2$ fraction. The 25% value agrees well with recovery data published for the β-receptor under similar conditions (31). An estimate of protein recovered after acetic acid elution indicates that only 0.025% of the amount applied to the column corresponds to the purified binding protein. The purified sample (estimated as 50 ng of protein) after acetic acid elution

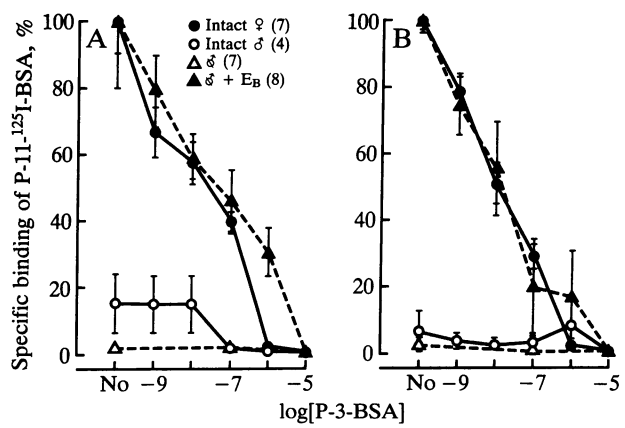


FIG. 3. Competition curves showing specific binding of P-11-$^{125}$I-BSA to crude membrane fractions ($P_2$) prepared from MBH–AHA–POA (*A*) and CS (*B*) tissues from adult male and female rats. "No" indicates specific binding when no competitor was present. Displacement after incubation of P-11-$^{125}$I-BSA with unlabeled P-3-BSA is shown. Specific binding was determined by subtracting nonspecific binding (cpm obtained in the presence of 10 μM P-3-BSA) from total cpm. Data are presented as percent of the value obtained in the absence of competitor for intact or castrate (♂) animals treated with $E_B$ or oil (see also Table 1). Data points are mean ± SEM for the number of experiments indicated in parentheses; SEM falls within symbol size where bars are absent.

Table 1. Comparison of specific binding of P-11-$^{125}$I-BSA and $K_i$ for P-3-BSA for hypothalamic (50 $\mu$g of protein) and CS (100 $\mu$g of protein) plasma membranes as a function of sex and hormonal condition

| Group | MBH–AHA–POA | | | CS | | |
|---|---|---|---|---|---|---|
| | *n* | Specific binding, cpm | $K_i$, nM | *n* | Specific binding, cpm | $K_i$, nM |
| Intact females | 7 | 6443 ± 517 | 52.2 ± 8.3 | 7 | 5052 ± 145 | 37.6 ± 8.7 |
| 7-day OVX | 5 | 7442 ± 515 | | 5 | 2745 ± 1393* | |
| 14-day OVX | 3 | 487 ± 245* | | 3 | 306 ± 85* | |
| 14-day OVX + oil | 6 | 835 ± 312* | | 6 | 350 ± 82* | |
| 14-day OVX + $E_B$ | 6 | 4218 ± 576 | 61.4 ± 9.9 | 6 | 5327 ± 1410 | 41.5 ± 9.2 |
| Intact males | 4 | 935 ± 557* | | 4 | 312 ± 300* | |
| Castrate males | 7 | 307 ± 204* | | 7 | 157 ± 133* | |
| Castrate + $E_B$ males | 8 | 6139 ± 1219† | 54.2 ± 3.6 | 8 | 3853 ± 1805† | 39.1 ± 7.5 |

Results are expressed as mean ± SEM for the number (*n*) of experiments indicated.
*Statistical significance by ANOVA as compared with the intact female condition, $P < 0.05$, or the 14-day OVX + $E_B$ group, $P < 0.05$.
†Statistical significance from intact males, $P < 0.01$, but no difference from intact females.

from the affinity column binds [$^3$H]P and the binding was specifically displaced by 10 $\mu$M free P (44 ± 4.5 vs. 5 ± 1 cpm, $n = 3$), which suggests that the eluted fraction contains a protein with affinity for P.

Finally, the results of SDS/PAGE before and after affinity chromatography are shown in Fig. 5 *B* and *C*, respectively. Before being applied to the affinity column, samples of the soluble fractions from $P_1$ and $P_2$ were lyophilized, resuspended in distilled water, and electrophoresed on a gradient gel under nonreducing conditions. Fig. 5*B* shows the profile of soluble proteins in these preparations as stained by Coomassie blue. Fig. 5*C* compares $P_1$ and $P_2$ fractions after affinity chromatography as stained by silver. SDS/PAGE shows a distinct band of ≈40–50 kDa in the $P_2$ lane that does not appear in the $P_1$ lane. Comparison of the proteins in the $P_2$ lanes before and after affinity chromatography suggests that the lighter band around the 50 kDa protein seen in Fig. 5*B* and the 40- to 50-kDa protein observed in Fig. 5*C* may be a neuronal membrane P-binding protein. Theoretical calculations indicate that the 40- to 50-kDa band after affinity chromatography is ≈0.025% of the protein added to the affinity column.

## DISCUSSION

The data presented here provide evidence of a specific sexually dimorphic membrane binding site for P in the rat CNS that may correspond to a protein of an estimated 40–50 kDa. The discrepancy in binding affinity between P-11-BSA and P-3-BSA agrees with previous reports (1, 14, 27). P-11-BSA binds with reduced affinity as compared to P-3-BSA and

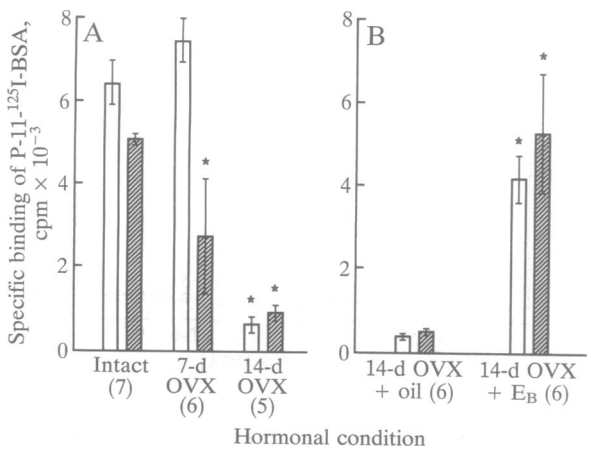


FIG. 4. Effect of ovariectomy (*A*) and $E_B$ replacement (*B*) on specific binding of P-11-$^{125}$I-BSA to $P_2$ fractions from adult MBH–AHA–POA (solid bars; 50 $\mu$g of protein) and CS (hatched bars; 100 $\mu$g of protein) tissues. Specific binding represents total binding (cpm) in the absence of inhibitor minus nonspecific binding. Data are mean ± SEM of the number of experiments indicated in parentheses. One-way ANOVA was performed to determine statistically significant differences in binding due to hormonal condition. *, Statistically significant difference from intact condition within the same brain region.

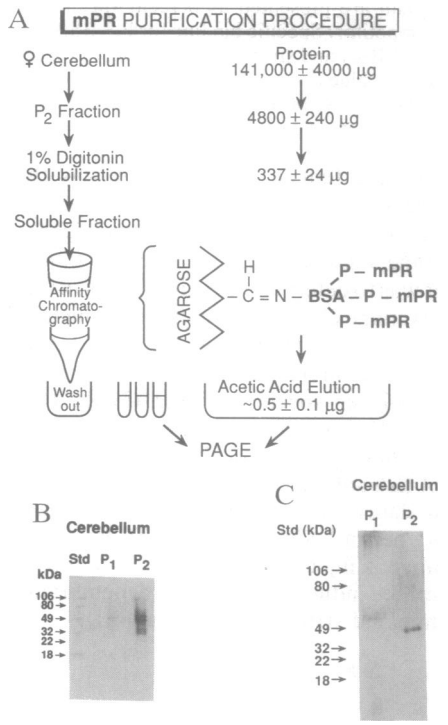


FIG. 5. Affinity chromatography procedure and results of SDS/PAGE. mPR, membrane P-binding protein. An example of the amount of protein (mean ± SEM, $n = 4$) in the homogenate, $P_2$ fraction, and soluble fraction is shown on the right of *A*. The latter fraction contains about 1.3 pmol/mg of protein as estimated from binding data. The protein recovered was 25% of the $P_2$ fraction. After resuspension of the lyophilized protein (before and after affinity chromatography) with 30 $\mu$l of distilled water, an 8-$\mu$l sample was applied to a 4–20% polyacrylamide gradient gel (*B* and *C*). SDS/PAGE was performed under nonreducing conditions. The soluble proteins in $P_1$ and $P_2$ prior to affinity chromatography and stained with Coomassie blue are shown in *B*. *C* shows the results of SDS/PAGE after affinity chromatography. Silver staining produces only a single band (≈40–50 kDa) in the $P_2$ lane, while no bands were detected in the $P_1$ lane. Standard markers as listed on the left side of the gels are as follows in kDa: 106, phosphorylase b; 80, BSA; 49.5, ovalbumin; 32.5, carbonic anhydrase; 27.5, soybean trypsin inhibitor; and 18.5, lysozyme. Since these are prestained standards (Sigma), the molecular mass is different from the true molecular mass of each standard molecule.

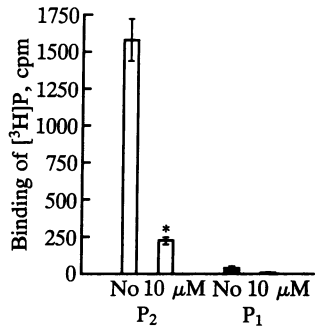


FIG. 6. Binding of [$^3$H]P to solubilized proteins (10 $\mu$g). "No" indicates binding in the absence of competitor; "10 $\mu$M" indicates binding in the presence of 10 $\mu$M P (nonspecific binding). Dimethyl sulfoxide at concentrations of 10% or less does not interfere in the assay. Data are presented as mean ± SEM for three experiments. *, Statistical significance ($P < 0.05$) as established by one-way ANOVA.

its usefulness in these studies is like that of receptor antagonists classically used in a variety of receptor binding assays. The high affinity displayed by P-3-BSA provides an appealing explanation for earlier findings, which have established that P-3-BSA stimulates the release of LHRH from superfused hypothalamic fragments, while P-11-BSA, given in equivalent doses, has no biological activity (20). Interestingly, P-11-BSA requires much larger doses than P-3-BSA to inhibit amphetamine-stimulated dopamine release, which agrees with its lower affinity, but it has similar potency to enhance amphetamine-stimulated dopamine release (26). One might speculate that the active site on the putative receptor is arranged such that it readily accepts the portion of the P molecule left exposed by conjugation with BSA at position 3, i.e., the side chain and rings C and D. For further discussion on this topic, refer to previous publications (2, 14, 27). Alternatively, placing the bulky BSA at position 11 might sterically hinder that same functional group from interacting easily with the active site on the membrane protein. An alternative explanation, which might explain discrepancies in total binding and affinities between the two conjugates, is that the membrane P-binding protein has multiple sites capable of interacting with P. One portion, a high-affinity low-capacity site, might interact with the part of P left exposed by conjugation at position 3. The other, low-affinity high-capacity, site might bind P-11-BSA, which might explain the differences in biological potency between these two conjugates.

In addition to showing ligand specificity, our results provide evidence that a membrane binding site for P demonstrates sexual specificity. This dimorphism is most likely attributable to differences in number or availability of receptors and probably not binding affinity. These data suggest that expression of the proposed membrane P-binding protein is dependent upon the hormonal environment of the animal.

Estrogen seems a logical candidate for a P-binding site-inducing substance, since estradiol has been shown to induce nuclear P receptors in certain brain regions (32–34) and in isolated cells in culture (35). Moreover, estrogen treatment is required for P-evoked LHRH and dopamine release and P-associated sexual behaviors. P enhancement of amphetamine-stimulated dopamine release is also established by estrogen treatment of castrated male rats, while P has no effect on amphetamine-stimulated dopamine release in intact males (22). That estrogen abolishes the sex difference in P binding to CS and hypothalamic membranes provides biochemical support for these biological findings.

P is known to interact either directly or indirectly with receptors for several neurotransmitter species (7, 10, 16). Herein we have described the purification of a specific membrane protein on the basis of its affinity for P. The purified protein appears to have a molecular mass of 40–50 kDa, which does not correspond to the molecular mass of other sites known to interact with P. This protein is specific to the membrane fraction, since it does not appear after affinity chromatography in the $P_1$ fraction. Although the $P_1$ fraction contains unbroken cells and a low concentration of membranes, these data agree with previous results that demonstrated no significant binding to $P_1$ fractions from cerebellum (14). Furthermore, preliminary results with a modified binding assay using the purified protein suggest that this protein binds [$^3$H]P. While the data presented here suggest that the 40- to 50-kDa protein is a membrane binding site for P, further investigation is necessary before it can be classified as a receptor. It is possible that the 40- to 50-kDa protein we have isolated is only a fragment of a larger binding molecule.

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