Binding of Steroids to Progesterone Receptor Proteins in Chick Oviduct and Human Uterus*

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SUMMARY

A number of synthetic and naturally occurring steroids were studied for competitive binding to two progesterone receptors, one in chick oviduct and another in human uterus. The results show a good correlation of relative (to progesterone) binding affinity with progestational activity as measured by subcutaneous Clauberg assay. 17α-Ethinyltestosterone and closely related compounds compete very well in binding to both receptors; 19-nor derivatives show dramatic enhancement of binding affinity to the human receptor. Of the substituted progesterones tested, 21-fluoro- and 6α-fluoroprogesterone competed best with progesterone for binding to both receptors. 17α-Acetoxyprogesterone and the substituted 17α-acetoxyprogesterones tested were fairly good competitors for the human receptor but did not compete at all for the chick. The binding data are assessed in terms of structural requirements for formation of a steroid-receptor complex. The data suggest that the receptors are able to modify the mode of binding at ring D to accommodate several different types of C-17 substitution.

Of the steroids with reactive halo, thio, diazo or chloromercuri substituents which were tested as possible substrates for affinity labeling of the receptor active site, only 6α- and 6β-bromoprogesterone and 17β-thiol- and 17β-methylthio-4-androsten-3-one competed sufficiently well with progesterone as to be considered good candidates.

Since the isolation of progesterone (4-pregnene-3,20-dione) from corpus luteum in the early 1930s, a variety of in vivo assays for progestational activity have been utilized, and the activities of some steroids have been found to vary widely depending on the nature of the assay and the manner of administration (1, 2). In some cases, the differences in progestational activity among steroids and the variations in activity of a particular progestogen have been attributed to metabolic transformation to a more or to a less active substance before reaching the target tissue (3). The possibility of metabolic transformation is influenced by the mode of administration (oral, subcutaneous injection, or uterine implantation). When administered orally, progesterone is rapidly inactivated and has a low progestational activity (1). Differences in absorption and binding to serum proteins may also contribute to the variability of results in in vivo tests. Thus a quantitative measure of progestational activity independent of whole body effects would be desirable.

The results of a number of in vitro investigations aimed at elucidating the sequence of events of target tissue response to a steroid hormone support the now generally accepted concept that an early event is the binding of the steroid at a highly specific site of a target tissue protein. Preliminary characterization and partial purification procedures have been reported for estrogen (4, 5), androgen (6, 7), and progesterone (8, 9) receptor proteins. A quantitative investigation of relative binding affinity of a number of steroids and nonsteroidal estrogens to the estrogen receptor protein in rabbit uterine cytosol was made by Korenman (10), who found a modest correlation of relative binding affinity with in vivo uterotrophic activity.

We have now undertaken competitive binding studies of a number of synthetic progestagens and related steroids to two progesterone receptor proteins, one found in chick oviduct cytosol and the other in human uterine cytosol. The progesterone-binding protein from chick oviduct has been investigated extensively and has properties indicative of a hormone receptor, including tissue and steroid-binding specificity, high binding affinity ($K_d \sim 10^{-9}$ to $10^{-10}$ M) and a low saturable concentration of binding sites (8, 9, 11, 12). Progesterone binding proteins having these properties have also been identified in mammalian uteri (13-16). Wiest and Rao (17) reported the existence of a protein in human uterus which binds progesterone with high affinity and specificity. Our initial studies confirm the results and demonstrate a protein in human uterine cytosol which binds progesterone with a dissociation constant of about $10^{-9}$ M. We have investigated the binding specificity of this receptor for comparison with that of the receptor in chick oviduct. Our aims
were to determine a possible correlation between relative binding affinity and progesterational activity by subcutaneous Clauberg assay, to assess the steroid structural and steric requirements for the formation of a steroid-receptor complex, and to find possible substrates for affinity labeling (18) of the receptor active site. Such a steroid substrate would have a high relative binding affinity and possess a functional group capable of forming a covalent bond with an amino acid residue within or adjacent to the active site (10, 20).

**EXPERIMENTAL PROCEDURE**

**Materials**

1. 2-3H [H]Progestosterone (48 Ci per mm) was obtained from New England Nuclear Corp. The radiochemical purity of this material was verified by thin layer chromatography in benzene-ethyl acetate (70:30). Sources of unlabeled steroids are indicated in the footnotes to Table I. Charcoal (Norit A), obtained from Sigma Chemical Co., was acid-washed and neutralized before use. Dextran 60 was from Pharmacia. All other chemicals were of reagent grade.

Female Rhode Island Red chicks were obtained from Acme Farms (Nashville, Tenn.) and were primed with daily subcutaneous injections of diethylstilbestrol (5 mg in sesame oil) for 10 to 20 days before use. Uteri were obtained from women who underwent elective hysterectomy during surgical repair of the pelvic region. All were normal cycling women between the ages of 25 and 41 years.

**Methods**

**Preparation of Cytosol Fractions**—Chick oviducts were removed, segmented, and rinsed in ice-cold 0.9% NaCl (saline) solution. They were then weighed and homogenized in 5 volumes (w/v) of Buffer A (0.01 M Tris-HCl-1.0 mM EDTA, pH 7.5), first with a Waring Blender followed by a Polytron PT-10 homogenizer (Brinkmann Instruments). The homogenate was centrifuged at 27,000 X g for 10 min and the supernatant fraction was recentrifuged at 120,000 X g for 1 hour to obtain the cytosol fraction. Very little loss in binding activity occurred during this time of storage.

**Preparation of Steroid Solutions**—The unlabeled steroids were prepared by combining 1 liter of the above solution with 500 ml of toluene-based scintillation fluid containing 42 ml of Spectrofluor Toluene (Amersham-Searle) per liter of toluene. The vials were let stand during 1 week of storage.

**Determination of Progestosterone Binding**—In all experiments, the binding of [3H]progestosterone was determined using the radiochemical adsorption method described in the footnotes to Table I. This method was used for both the chick and human cytosol fractions. The chick cytosol was diluted either 5- or 10-fold with Buffer A before use and the human cytosol was used without further dilution. Aliquots (0.3 ml) of cytosol were added to assay tubes containing 20,000 to 30,000 cpm of [3H]progestosterone in 0.05 ml of Buffer A and 0.1 ml of Buffer A alone or containing various concentrations of unlabeled steroid competitors. The assay ingredients were mixed and the tubes were incubated for 18 to 20 hours in an ice bath. At the end of the incubation, 0.5 ml of charcoal suspension (0.5% Norit A and 0.005% Dextran 60 in Buffer A, pH 7.5) was added to each tube. The tubes were briefly agitated and incubated for an additional 10 min at 0°C. They were then centrifuged for 10 min at 500 x g, and the clear supernatants were quantitatively extracted into counting fluid which was prepared by combining 1 liter of the above solution with 500 ml of Triton X-100. The counting efficiency was approximately 30%.

**Preparation of Steroid Solutions**—The unlabeled steroids were progesterone from the aqueous phase into the toluene. Counting efficiency was approximately 30%. In some cases, the aqueous sample was dissolved in toluene-based scintillation fluid which was prepared by combining 1 liter of the above solution with 500 ml of Triton X-100. The counting efficiency was approximately 30%.
carefully weighed and dissolved in dimethylsulfoxide to a concentration of 0.1 mg per ml. In a few experiments, ethanol was used as the initial solvent. The desired steroid concentrations were prepared by serial dilution of the initial solution with Buffer A. In all cases, the final concentration of solvent (dimethylsulfoxide or ethanol) in the assays was less than 0.5% and did not affect steroid binding. The steroid solutions were prepared within 1 day of analysis and fresh solutions were used for each test of a given competitor.

**Competitive Binding Assay**—In each experiment, a series of assay tubes was prepared containing 0.5 ml of cytosol plus 0.15 ml of [°H]progesterone and unlabeled steroid competitors in Buffer A as described above. The procedures and method of competitor analysis are based on those of Korenman (10). The assay tubes were incubated at °C for 16 to 20 hours and [°H]progesterone binding was measured by charcoal adsorption. Each assay point was determined in duplicate. A standard curve for the competition of five progesterone concentrations in the range of $3.5 \times 10^{-6}$ M to $7.1 \times 10^{-4}$ M. Four or five concentrations of each competitor were tested using a range between $3 \times 10^{-4}$ and $7 \times 10^{-2}$ M. The competitor concentrations were chosen to provide a linear portion on a semilog plot which would cross the point of 50% competition (see Figs. 1 and 2). From this plot, the concentrations of unlabeled progesterone and of steroid competitors that reduced °H]progesterone binding by 50% were determined. These quantities were converted to molar concentrations and the effectiveness of a competitor was established using the ratio of unlabeled progesterone concentration for 50% competition to competitor concentration for 50% competition. This ratio was multiplied by 100 and termed the RA. Therefore, by definition, the RA of progesterone equals 100.

1 The abbreviation used is: RA, relative binding affinity.

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**Problems in Competitor Analysis**—A problem encountered in the evaluation of certain competitors with human uterine cytosol (but not in experiments using chick oviduct cytosol) is illustrated in Fig. 3. In the experiment shown, unlabeled progesterone produced a good competition curve, but the competing ability of 17α-ethyl-19-nortestosterone (IV-12) appeared to be incomplete. However, when a noncompeting quantity of unlabeled cortisol was added to the assay tubes, 17α-ethyl-19-nortestosterone was found to be an effective and complete competitor. This phenomenon occurred to a greater or lesser extent in most human cytosol preparations, but only with certain competitors, notably 17α-substituted progesterones and 19-nortestosterone derivatives. Most other steroids were completely unaffected by the presence of cortisol. The effect of cortisol remains unexplained, but one possibility is that there are other steroid-binding components in the cytosol which can reduce the effectiveness of certain competitors. Cortisol may bind to these components and eliminate their interference in the competitive binding assay. In any case, when this phenomenon occurred, the results were discarded and the assay was repeated in the presence of $5 \times 10^{-4}$ M cortisol.

**RESULTS**

Figs. 1 and 2 show typical competitive binding curves for a few of the steroids whose relative binding affinities are summarized in Tables II to IV. The compounds have been listed in the three tables according to structural similarity. In most cases at least two and frequently more assays were made for each competitor. Agreement between multiple assays was generally good. However, in a few tests rather wide variations occurred (II-15, II-19, and III-30).

For comparison with these competitive binding studies the available progestational activities of the steroids as determined by classical Clauberg assay are also listed in Tables II to IV. As noted earlier progesterone is not highly active in oral tests, and 17α-ethyltestosterone is the reference compound (50) for these data (see Footnote 4 to the tables). Thus subcutaneous tests provide a more meaningful measure of progestational activity, but the results of oral tests are relevant to actual use of the compounds as progestagens. It should be noted that the Clauberg assay is a measurement of endometrial proliferation and is not necessarily indicative of the effect of progesterone in pregnancy maintenance. Certain compounds with high Clauberg activity do not maintain pregnancy in ovariectomized animals and inhibit normal pregnancies in intact animals. According to Madjerek (51), however, compounds which do maintain pregnancy never lack a progestational effect on the endometrium.
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<sup>a</sup> Relative binding affinity, progesterone = 100.  
<sup>b</sup> Subcutaneous administration, progesterone = 100.  
<sup>c</sup> Number in parentheses is literature reference.  
<sup>d</sup> Oral administration, ethisterone (17α-ethynyltestosterone) = 100.  
<sup>e</sup> These activities are reported in the literature with several different compounds as reference. All oral activities in this table have been normalized to the single reference compound.  
<sup>f</sup> Intrauterine administration, progesterone = 100.  
<sup>g</sup> Private communication from Roy Hertz, New York Medical College, Valhalla, New York.
hydrocarbon (saturated or unsaturated) substituent. There appears to be a good qualitative correlation of competitive binding to the human receptor with Clauberg activity in both these classes: compounds that bind as well or better than progesterone have at least as high or higher activity, and with the exception of 11β-hydroxyprogesterone (II-11), there are no good binders with poor Clauberg activity. A graphic summary of the data illustrating this correlation is shown in Fig. 4. In the case where a poor binder, (α)-17α-chloroethyl-18-methyl-19-nortestosterone-3β,17β-diol 3-acetate (1V-16), shows extremely high Clauberg activity, it is likely that the steroid is transformed to a potent compound in vivo. In cases where the Clauberg activity is extremely high and the competitive binding fair, notably 17α-acetoxy-6-chloro-Δ4-progesterone (II-16) and 17α-acetoxy-4,6-dichloro-Δ4-progesterone (II-17), the high Clauberg activity may be a consequence of resistance to metabolic transformation. The double bond at C-6 and the 4-chloro substituent may substantially retard reduction to less active substances. Alternatively, these steroids may bind to other cytosol components thereby reducing the concentration available for competition at the progesterone binding site. In the case of 11β-hydroxyprogesterone (II-11), a relatively good binder, the low Clauberg activity may represent a rapid metabolic inactivation.

In the case where both isomers, 6α- and 6β-bromoprogesterone (II-4 and II-5), were evaluated in parallel, the 6β isomer produced a large increase in progesteragenic activity as measured by Clauberg assay (II-13 and 11-14) and also has a similar effect on competitive binding to the human receptor (increase of RA from 3 to 40). The substituted 17α-acetoxyprogesterones (II-15 to II-18), all extremely potent progestagens by Clauberg assay (II-13 and 11-14) and also has a similar effect on competitive binding to the human receptor (increase of RA from 3 to 40). The substituted 17α-acetoxyprogesterones tested (II-15 to II-18), all extremely potent progestagens by Clauberg assay (II-13 and 11-14) and also has a similar effect on competitive binding to the human receptor (increase of RA from 3 to 40). 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IV-20 to IV-22) were tested to assess their potential to form covalent bonds with receptor sulfhydryl groups at the active site. Not surprisingly, natural (estrone, estradiol) and synthetic (dichotylstilbestrol) estrogens showed no competition for either receptor.

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**DISCUSSION**

Early hypotheses for the mechanism of action of steroid hormones dealt mainly with consideration whether the α or the β face of the steroid was involved in steroid-receptor interaction. Recently Vida (56) has presented an extensive analysis of data relating structural modification with androgenic activity and based upon it has postulated a three-dimensional outline of this steroid-receptor complex involving bonding at the α face of ring A, the β face of rings B and C, both α and β faces of ring D, and peripheral bonding in planes perpendicular to the α and β faces of the steroid.

The binding data presented here do not allow a complete outline of the progesterone-receptor complex, but a partial picture is possible. After summarizing the general differences between the human and chick receptors, a model for the receptor site, similar for both proteins, will be discussed. Except in the case of a 17α-acetoxy substituent, which it emphatically rejects, the chick receptor appears to tolerate structural variations better than the...
human, which in turn appears more selective. Examples are saturation of the C-4 double bond and conversion of the C-3 carbonyl to an α- or β-hydroxyl group (III-1 to III-4), and C-21 functionalization (Ill-19 to Ill-23). 19-Norsteroids generally bind somewhat better to the chick receptor than do their C-10 methyl counterparts, but the improvement in binding to the human receptor is dramatic (see Table V).

For the human receptor, removal of the 19-methyl group is the only modification of ring A which does not decrease or eliminate receptor binding. The steric requirement for ring A appears secondary in importance to the α,β-unaturated carbonyl system, but both are substantial. Reduction to the sterically similar (A/B trans) 5α-dihydroprogesterone (III-1) results in a RA decrease to 12; the RA of the A/B cis 5β isomer (III-2) is not greatly lower (RA 6). Flattening of the A (or A/B) ring by the introduction of a second double bond at C-1 or C-6 (II-30 and II-31) reduced the RA to only about 50 in either case. These observations suggest an important area of bonding at the β face of rings A and B, and α face hydrogen bonding at the C-3 carbonyl group which is weakened when the carbonyl group is tilted upward. The fact that 11β-hydroxyprogesterone (II-11) binds fairly well (RA 36) while 11α-hydroxyprogesterone (II-10) is a poor competitor (RA 1) suggests peripheral binding at C 11 which is disrupted by the equatorial substituent.

The human receptor appears to tolerate structural modifications of ring D better than those of ring A. Conversion of the C-20 carbonyl to an α-hydroxyl group reduces the RA to 40, but the 17β-acetyl side chain is not required for maximum binding if both a 17β-hydroxyl group and a 17α-alkyl or alkynyl side chain are present. Thus the receptor appears able to modify its mode of binding at ring D to accommodate two different types of C-17 substitution. Let us first consider the 17α-alkyl-17β-hydroxyl mode. The high binding affinity of 17α-alkyl-18-methyltestosterones (IV-13a, II-14, and IV-15) rules out significant β face interactions, and the binding will be associated with the 17α-substituent below the α face, and peripherally at the 17β-hydroxyl group.

Liao's recent study (57) of competitive binding of steroids to the rat ventral prostate androgen receptor indicates some similarities in binding to this receptor and to the progesterone receptors studied here. Comparison of the results shows that two structural modifications of a steroid, 17α substitution or the absence of a C-19 methyl group generally increase, or at least do not decrease, binding affinity for each of the three receptors.

Molecular orbital calculations have indicated that the preferred conformation for the side chain of 20-oxopregnanes is that in which the C-20 carbonyl group projects toward the β face of ring D, and the C-20 to C-21 bond eclipses the 17α-C—H bond (58). This has recently been confirmed by x-ray analysis (59). Thus some β face interaction associated with bonding to the C-20 carbonyl group is likely. For the human receptor, introduction of a 17α-acetoxyl group reduces, but sometimes only slightly, binding relative to progesterone. Thus α face interaction is probably not very important in the mode of binding for progesterones, but some interaction with a 17α-acetoxyl substituent may take place when one is present, perhaps an interaction similar to that proposed for the mode of D ring binding for 17α-alxytestosterones. The observation that the chick receptor, in contrast to the human, will not bind 17α-acetoxyprogesterones suggests an important structural difference between the two proteins in the region involved in D ring binding. Alternatively, the 17α-acetoxyprogesterones may bind strongly to other receptor sites present in chick oviduct cytosol, thereby reducing the concentration available for competition at the progesterone binding site.

The enhanced binding observed with 6α-fluoro- and 21-fluoroprogesterone (II-3 and II-19) warrants comment and several explanations are possible. Inductive effects would reduce hydrogen bonding at the respective carbonyl groups and are probably not important. However, the relatively high ionic character of the C—F bond due to the high electronegativity of the fluoride atom may allow strong hydrogen bonding between the fluoride atom and a proton donor group of the receptor. Such an interaction has been proposed (60) to explain the enhanced Clauberg activity of 21-fluoroprogesterone, and infrared data in support of the proposal were given. Alternatively, the presence of fluoride atoms α to a carbonyl group is known to stabilize the α-hydroxy-

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**Table V**

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<th>Compound</th>
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**FIG. 4.** Relative binding affinity to human uterine cytosol for various progestagens correlated with their respective progestational activity by Clauberg assay (subcutaneous except for II-SB and II-36 which are by intrauterine administration). ---, a correlation coefficient of +1.
Sulfur hydride (hemithioketal group) formed by reaction with a sulfur hydride compound (61) (Reaction 1). Since sulfur hydride blocking agents have been shown to destroy progesterone binding activity

\[
\text{II-19} + \text{HS receptor} \rightarrow \text{II-19} + \text{O}
\]

(8), sulfur hydride groups may exist at or adjacent to the active site. If this is true, the enhanced binding observed with fluoroprogesterones II-3 and II-19 may be due to the formation of a more stable α-hydroxysulphide as shown in Reaction 1. The negative effect of a second 21-fluoro substituent (11-20) on binding might then be steric in nature.

Our picture of the receptor active site implies envelopment of the steroid by the receptor and hence extensive conformational changes in the protein. The picture is in agreement with the observed slow rate of formation of the complex and the slow rate of dissociation once it is formed (9).

In conclusion, the present results provide a reasonably good correlation between protein binding and progestational Cmaberg activity, and thus offer additional evidence for a function of the steroid by the receptor and hence extensive conformational changes in the protein. The picture is in agreement with the observed slow rate of formation of the complex and the slow rate of dissociation once it is formed (9).

Acknowledgments—We thank Drs. David J. Ellis and Howard J. Ringold, Syntex Research, and Dr. Evelyn S. France, Wyeth Laboratories, for generous gifts of steroids, Professor Roy Hertz, New York Medical College, for Clauberg assay data, and Suzau R. Pfeifer and Ross C. Hardison for technical assistance.

References


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