Kinetic and physicochemical characteristics of an endogenous inhibitor to progesterone–receptor binding in rat placental cytosol

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This study describes the kinetic behaviour and physicochemical aspects of an endogenous inhibitor of progesterone–receptor binding in trophoblast cytosol from day-12 embryos. The progesterone cytosol receptor was partially purified and isolated from the inhibitor as the 0–50%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction. The inhibitory substance was shown to reside in the 50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction. Equilibration of the inhibitor preparation with the receptor fraction increased the K_D^{app} of the ligand–receptor binding reaction in a concentration-dependent manner (26 ± 3 -fold increase in K_D^{app} per mg of protein of the $(\text{NH}_4)_2\text{SO}_4$ fraction, $n = 16$). However, the inhibitor did not alter the concentration of binding sites. Studies of other physicochemical aspects of the inhibitor showed it to be non-diffusible, excluded from Sephadex G-25, stable at 35°C for 30 min, but irreversibly denatured at 70°C for 30 min. The Stokes' radius was estimated by gel chromatography to be 2.8 ± 0.11 nm ($n = 5$). Inhibitory activity was destroyed by HgCl_2 , suggesting that disulphide bridges play an essential role in the biological activity of this molecule. The inhibitor is a macromolecule which does not bind progesterone and differs from albumin. The kinetic mechanism by which the inhibitor enhanced K_D^{app} was investigated by measuring association and dissociation rate constants and the energy of activation (E_a) for each reaction. The association rate (k_+) for progesterone and receptor was $(1.3 \pm 0.2) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ but declined to $(0.4 \pm 0.1) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($n = 5$) when exposed to the inhibitor ($P < 0.01$). The dissociation rate (k_-) was $(3.2 \pm 0.6) \times 10^{-5} \text{ s}^{-1}$ for progesterone–receptor complex and was unchanged by the inhibitor. The E_a for the association of complex was 33.6 ± 4.2 kJ/mol and was increased to 63.0 ± 8.4 kJ/mol by the inhibitor ($P < 0.05$). The E_a of dissociation was unaltered. Thus, an inhibitor is present in trophoblast cytosol which specifically enhances K_D^{app} without altering availability of binding sites. The mode of action of inhibitor is to increase the energy of activation for association of complex without influencing the dissociation reaction.

The interaction between progesterone and its cytosol receptor is an acknowledged primary event in progesterone-responsive tissues and leads to translocation of the complex to the nucleus with concomitant depletion of receptor from the cytosol. This process culminates in elicitation of the physiological events typically ascribed to the hormone. Within several hours replenishment occurs and the cytosol receptor approaches its original concentration. These phenomena have been investigated in rat uterus (Feil *et al.*, 1972; Hsueh *et al.*, 1974; McGuire & Bariso, 1972; Walters & Clark, 1977, 1978). Recently, a progesterone receptor has been identified and characterized in rat trophoblast (Ogle, 1980). The trophoblast receptor exhibits physiological properties such as depletion and replenish-

ment, as has been observed for other receptor systems. Furthermore, concentration of receptor is dependent on oestradiol, as for other progesterone-responsive systems (Evans *et al.*, 1978; Li & Li, 1978; Mester *et al.*, 1979). During the study of these processes it was discovered that dilution of cytosol enhanced the affinity of progesterone for receptor but did not alter the concentration of binding sites (Ogle, 1981). This suggested that an endogenous inhibitory substance was present which interacted specifically with the ligand–receptor binding rather than behaving as a class of low-affinity binder. The present work was undertaken to isolate the inhibitory material from cytosol receptor protein and to examine the kinetic behaviour of this ligand–receptor–inhibitor system.

Materials and methods

Preparation of placental cytosol

Female Long-Evans rats (225–250 g) were maintained under a constant photoperiod of 12 h light/12 h dark and a temperature of 22°C. All animals received food and water *ad libitum*. Day 1 of pregnancy was determined by appearance of vaginal sperm on the morning following overnight exposure to fertile males. Rats were killed by decapitation between 07:00 and 08:00 h on day 12 of pregnancy. Uteri were excised and placed in ice-cold TDG 30% buffer [0.01 M-Tris/HCl/30% (v/v) glycerol/1.0 mM-dithiothreitol], pH 7.8 at 4°C. Each uterus was sliced and everted, thereby exposing the amnionic membranes, the embryos, and placentae. The placental trophoblast was carefully separated from the former tissues and the underlying maternal tissue. Histological examination revealed that the preparation contained the spongiotrophoblast (giant and small cytotrophoblast cells) and very little labyrinth tissue. Trophoblast tissue was pooled and homogenized at 0°C in a hand-driven glass homogenizer. Ice-cold conditions (0°C) were maintained throughout subsequent procedures.

Exchange assay under equilibrium conditions

The homogenate was centrifuged at 800 g for 15 min at 4°C. The supernatant was removed and treated with a suspension of 0.5% charcoal/0.05% dextran in TDG 30% at 0°C to strip cytosol of endogenous steroids (Ogle, 1980). After 10 min the mixture was centrifuged at 157 000 g for 50 min. The resultant supernatant served as cytosol for subsequent experiments.

A sample (100 µl) of the placental receptor preparation (0.2–0.3 mg of protein) was incubated with 200 µl of TDG 30% containing six concentrations of [³H]progesterone (0.8–22 nM) in the presence or absence of a 100-fold molar excess of unlabelled progesterone at 4°C for 20–24 h. Control conditions were provided by incubating ligand and receptor without inhibitor. An additional set of tubes was utilized to ascertain activity of various inhibitory preparations. Aliquots (100 µl) of inhibitor were added to each tube containing identical concentrations of ligand and receptor; the volume of buffer was decreased accordingly such that each reaction tube contained an equal volume. Bound [³H]progesterone was separated from free [³H]-progesterone by addition of 700 µl of dextran/charcoal in TG10% buffer [0.01 M-Tris/HCl/10% (v/v) glycerol] at 0°C for 5 min. Incubation with dextran/charcoal was terminated by centrifuging for 15 min at 1600 g. A sample (600 µl) was transferred to vials containing 3 ml of toluene scintillation cocktail and counted for radioactivity.

Equilibrium binding parameters were calculated

by the direct linear plot (Cornish-Bowden & Eisenthal, 1974), the double-reciprocal plot, and the Scatchard plot (Segel, 1975). The former was carried out by a computer program written in FORTRAN and fits experimental data to the Michaelis-Menten equation according to the median method (Cornish-Bowden & Eisenthal, 1974), and modified according to the procedure of Porter & Trager (1977) and Cornish-Bowden & Eisenthal (1978). The double-reciprocal plot and Scatchard analysis allow detection of co-operativity and the number of binding moieties by their deviation from linearity (Rodbard, 1973). Equilibrium parameters estimated by these methods were essentially in agreement.

Protein concentration of each cytosol preparation was determined by the method of Lowry *et al.* (1951). Bovine serum albumin (Cohn fraction V) was utilized as standard.

Chemicals

Unlabelled progesterone, HgCl₂, *N*-ethylmaleimide and dithiothreitol were purchased from Sigma. [1,2,6,7-³H(N)]Progesterone, 90–114 Ci/mmol, was purchased from New England Nuclear. The labelled steroid was purified before use on a Sephadex LH-20 column (0.5 cm × 16 cm) in benzene/methanol (17:3, v/v).

Cytosol fractionation by precipitation with (NH₄)₂SO₄

Cytosol was fractionated by differential precipitation with (NH₄)₂SO₄ [a saturated solution of (NH₄)₂SO₄ in 0.01 M-Tris at 0°C and adjusted to pH 7.8 with 0.1 M-NH₄OH]. Cytosol was brought to the desired level of saturation by slow dropwise addition of the saturated solution with constant stirring. After 1 h incubation at 0°C, the cytosol was centrifuged at 50 000 g at 4°C for 10 min to sediment the precipitate. If necessary, the supernatant was transferred to another ultracentrifuge tube at 0°C for precipitation of additional fractions. Pellets were redissolved in TDG 30% at a concentration equivalent to 150–200 mg of placental tissue/ml.

Gel filtration

The molecular size of the cytosol inhibitory substance was estimated by column chromatography using Sephadex G-75 (Pharmacia). A column of 1.6 cm × 30.0 cm was washed extensively with TDG 30% buffer. Calibration was carried out by determining the elution volumes (*V*_e) of three highly purified protein standards of known Stokes' radius: ovalbumin, 2.76 nm; chymotrypsinogen A, 2.25 nm and ribonuclease A, 1.80 nm (Tanford, 1961). Blue Dextran 2000 was utilized to determine the void volume (*V*₀). *V*₀ and all *V*_e values were estimated in 0.45–0.50 ml samples applied to a 60 ml

column (V_T) eluted by upward flow at a rate of 36 ml/h at 18°C. K_{av} , $2(V_e - V_0)/(V_T - V_0)$ was calculated for each standard and plotted as $(-\log K_{av})^{1/2}$ versus Stokes' radius. The standards fell within the linear portion of the fractionation range of Sephadex G-75. The column was calibrated at the initiation of the experiments and again upon their completion. Values for V_0 , V_e and K_{av} were essentially invariant between this interval. The $(-\log K_{av})^{1/2}$ of the inhibitory material was estimated for five pools of cytosol and compared to the $(-\log K_{av})^{1/2}$ versus Stokes' radius relationship obtained for the protein standards (Siegel & Monty, 1966).

Association rate kinetics

Whole cytosol or the 0–45%-satd. $(NH_4)_2SO_4$ fraction were utilized as the receptor preparation in these experiments. All reactions were performed at 0°C unless otherwise noted. The reaction mixture contained an appropriate concentration of $[^3H]$ -progesterone in TDG 30% in the presence or absence of inhibitor; volume was adjusted to 1.0 ml with buffer. At time zero, 1.0 ml of the receptor preparation (1–2 mg/ml) was added to the mixture. At 0.5–2 min intervals thereafter, aliquots (100 μ l) were withdrawn to tubes containing a 100-fold molar excess of unlabelled progesterone in 0.5 ml of TDG 30%. The excess unlabelled progesterone was employed to prevent further association of $[^3H]$ -progesterone with receptor. Upon completion of the reaction, $[^3H]$ -progesterone remaining free was adsorbed to 300 μ l of dextran/charcoal. The amount of ligand bound to receptor was determined by scintillation spectrometry.

Initial progesterone receptor concentration was quantified by the exchange assay under equilibrium conditions as described above. This value was employed in the calculation of the association rate constant for each receptor preparation.

Dissociation rate kinetics

The dissociation process of the hormone–receptor complex in whole cytosol and partially purified cytosol preparation [0–45%-satd. $(NH_4)_2SO_4$ fraction] was studied by the exchange assay method.

The cytosol preparation was equilibrated with excess $[^3H]$ -progesterone for 18–20 h at 0°C and treated with dextran/charcoal to remove unbound steroid. The dextran/charcoal was sedimented by centrifugation after a 5 min incubation at 0°C. The supernatant was decanted into a glass test tube containing a 100-fold molar excess of unlabelled progesterone to prevent reassociation of liberated $[^3H]$ -progesterone with receptor. An aliquot was analysed for zero-time concentration of hormone–receptor complex. These procedures were complete in less than 2% of the half-life of the complex. Thus dissociation was insignificant relative to obtaining the zero-time sample. At appropriate time intervals, aliquots (200 μ l) were placed with 600 μ l of 0.17% charcoal/0.0017% dextran suspended in TG 10%. The dissociation reaction was followed for 54 h at 0°C in an ice bath. The stability of receptor throughout this period has been established (Ogle, 1980). Kinetic characteristics of dissociation were studied with regard to the number of dissociating moieties according to the procedures recommended by Rodbard (1973).

Results

Separation of the endogenous inhibitor from the cytosol receptor

Previous experiments demonstrated that the affinity of progesterone–receptor binding was inversely proportional to the concentration of cytosol (Ogle, 1981) suggesting that an endogenous inhibitory substance was present in cytosol. An attempt was made to separate the inhibitor from the high affinity binding component by a procedure based on the thermolabile nature of the receptor (Ogle, 1980). Cytosol was incubated in a water bath at 35°C for 30 min. The heat-treated denatured cytosol preparation was then centrifuged for 15 min at 1600 g and the supernatant was assayed for inhibitory activity. It exhibited about 6% of the high affinity binding sites measured in samples of the same cytosol preparation not exposed to the heat treatment, but when co-incubated with the latter it enhanced the apparent (K_D^{app}) 13 ± 3 -fold over the control ($n = 4$, $P < 0.01$). Table 1 indicates that, for

Table 1. *Effect of an endogenous inhibitor on kinetics of progesterone binding in cytosol from rat trophoblast*

Whole cytosol (mg of protein/tube)	Inhibitor preparation* (mg of protein/tube)	K_D^{app} (nM)	B_{max} (fmol of binding sites/mg of protein)
0.86	—	7.35	275
0.42	—	4.39	247
0.42	0.44	6.99	287
0.24	—	3.69	236
0.24	0.26	6.17	347

* Aliquot of whole cytosol incubated at 35°C for 30 min to eliminate high-affinity binding.

each decrease in the concentration of whole cytosol, there was an accompanying decline in K_D^{app} , which indicates the presence of an endogenous inhibitor. However, co-incubation of heat treated cytosol with whole cytosol reversed the influence of dilution and K_D^{app} exhibited an increment at each concentration without changing the availability of receptor binding sites. Taken together these observations suggested that the inhibitory substance was distinct from the progesterone receptor moiety and did not bind progesterone; thus it is not merely another class of binder with lower affinity for progesterone.

These findings suggested that it should be possible to divide receptor and inhibitor into separate fractions. The progesterone receptor fraction should exhibit kinetics similar to that of dilute whole cytosol; however, when co-incubated in the presence of inhibitor, progesterone should exhibit a decrease in affinity for receptor. To this end, cytosol was partitioned by differential precipitation with a saturated solution of $(NH_4)_2SO_4$ as described in the Materials and methods section with the object of isolating a fraction rich in receptor and one rich in

Fig. 1. Effects of various cytosol fractions prepared by $(NH_4)_2SO_4$ precipitation on progesterone-receptor interaction

Aliquots of whole cytosol were utilized as a control (kept at 0°C), or treated with a saturated solution of $(NH_4)_2SO_4$, or incubated in a 35°C water bath for 30 min. Aliquots from each treatment group (0.3–0.6 mg of protein/tube) were then added to the control cytosol (0.3 mg of protein/tube) and allowed to reach equilibrium overnight with $[^3H]$ progesterone (0.8–22 nM) $\pm$ a 100-fold excess of unlabelled progesterone. $\circ$, Whole cytosol; $\bullet$, cytosol + 0–30% satd. $(NH_4)_2SO_4$ precipitate; Δ , cytosol + 30–53% satd. $(NH_4)_2SO_4$ precipitate; $\blacktriangle$, cytosol + 53–64% satd. $(NH_4)_2SO_4$ precipitate; $\square$, cytosol + heat-treated cytosol.

inhibitory activity. The inhibitory activity of each fraction was assayed by incubating aliquots (100 μ l) with whole cytosol at four to six concentrations of $[^3H]$ progesterone (0.8–22 nM); binding constants were determined by the equilibrium exchange assay procedure. Fig. 1 summarizes the results from a representative experiment. K_D^{app} was unaffected by various cytosol fractions prepared by $(NH_4)_2SO_4$ precipitation until the cytosol was brought between 53 and 64% saturation. This fraction enhanced K_D^{app} more than 6-fold with no change in the concentration of specific binding sites (B_{max}). Heat treatment of an aliquot of the whole cytosol exhibited essentially identical behaviour.

The next experiments showed that 100% of the high-affinity sites were precipitated when cytosol reached 50% saturation with $(NH_4)_2SO_4$ and that inhibitory activity was confined to those substances which precipitated between 50 and 70% saturation

Fig. 2. Summary of binding properties of various cytosol fractions prepared by $(NH_4)_2SO_4$ precipitation. Inhibitor activity was assayed after overnight equilibration with ligand and receptor (0–40% satd. fraction). Values are means $\pm$ S.E.M.; n = number of experiments.

(Fig. 2). Each of the two main salt fractions (0–50% and 50–70%-satd.) was subdivided into smaller fractions to examine with greater resolution the precipitation of receptor and inhibitor. The precipitate of the 0–40% fraction contained essentially all of the receptor and exhibited the same K_D^{app} as did whole cytosol. The precipitate of the 40–50%-satd. fraction also contained receptor and exhibited the same kinetic behaviour as the 0–40%-satd. fraction. The 50–70%-satd. fraction increased the K_D^{app} of progesterone–receptor binding, but exhibited no specific binding properties by itself. Subfractions were inhibitory but less potent. Non-specific binding increased dramatically in fractions greater than 70%-satd. and thus it was not feasible to go beyond this limit.

Incubation of the 0–40%-satd. fraction with the 40–50%-satd. fraction enhanced the number of receptor binding sites by 147% but did not alter K_D^{app} , whereas incubation with the 50–70%-satd. fraction enhanced K_D^{app} almost 2.5-fold with no change in B_{max} . (Fig. 3). These findings are consistent with the notion that the 0–50%-satd. fraction was rich in receptor but not in inhibitor. Thus, in subsequent experiments the 0–45%-satd. fraction was used as the receptor fraction and the 50–70%-satd. fraction served as the inhibitory fraction. The 45–50%-satd. fraction was discarded as a buffer zone to minimize cross-contamination of the respective progesterone receptor and inhibitor preparations. Further characterization of the kinetic influence of the inhibitory fraction revealed that enhancement in K_D^{app} was concentration-dependent, having a potency to increase K_D^{app} 26 ± 3 -fold

per mg of protein (16 experiments). However, concentration of receptor binding sites was independent of inhibitory concentration. The results of a representative experiment are shown in Fig. 4.

Physical evidence that the inhibitory preparation (50–70%-satd. fraction) did not contain progesterone binding sites was obtained by sucrose density gradient analysis of receptor–inhibitor interaction. Fig. 5 presents representative results from several experiments which clearly show that all specific binding was confined to the receptor fraction (0–45%-satd.); the inhibitor fraction (50–70%-satd.) contained no high affinity binding sites for ligand nor did it alter the concentration of high affinity sites in the receptor fraction. A rather broad binding peak occurred in the 4S region when the inhibitor was incubated with receptor but binding was entirely due to non-specific interactions.

That the kinetic characteristics between the partially purified cytosol preparations $[(NH_4)_2SO_4]$ precipitates and the crude preparations (dilution of whole cytosol and heat-treated cytosol) were similar offers evidence that the same reaction is under scrutiny and that there is a specific interaction of hormone–receptor–inhibitor which is influenced only by a specific component(s) present in cytosol. All of these findings, taken together, support a

Fig. 3. Separation of cytosol into receptor-rich and inhibitor-active fractions by $(NH_4)_2SO_4$ precipitation. All fractions were prepared from the same cytosol pool. The 0–40%-satd. fraction served as receptor (0.1 mg of protein/tube). O, Control (0–40%-satd. fraction); ●, 0–40% + 40–50%-satd. fraction; △, 0–40% + 50–70%-satd. fraction.

Fig. 4. Effects of inhibitor concentration on equilibrium parameters of progesterone–receptor binding.

The receptor [0–45%-satd. $(NH_4)_2SO_4$ fraction] was incubated overnight with various concentrations of the inhibitor (50–70%-satd. fraction). All fractions were prepared from the same pool of cytosol. ●, K_D^{app} (nm). O, B_{max} . (pmol/mg of receptor protein).

Fig. 5. Sucrose gradient analysis of the trophoblast cytosol progesterone receptor and inhibitor

Receptor [0–45%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction] $\pm$ inhibitor (50–70%-satd. fraction) in TDG 10% buffer was incubated with $[\text{H}^3]$ progesterone (15 nM) $\pm$ a 100-fold molar excess of unlabelled progesterone for 3 h at 0°C , and then layered onto 4 ml 5–20% (w/v) linear sucrose gradients prepared in TDG 10% containing $[\text{H}^3]$ progesterone (8 nM) $\pm$ a 100-fold molar excess of unlabelled progesterone. Centrifugation was performed at 156000g for 16 h at 4°C . The gradients were fractionated into 100 μl aliquots at 4°C , and unbound $[\text{H}^3]$ progesterone was removed by dextran/charcoal adsorption. Standards of ovalbumin (3.6S) and bovine γ -globulin (6.6S) were layered on similarly prepared gradients and centrifuged concurrently. Sedimentation coefficients were calculated and are indicated by arrows (Martin & Ames, 1961). Receptor only: $\bullet$, total binding; $\circ$, non-specific binding. Receptor + inhibitor: $\blacktriangle$, total binding; $\triangle$, non-specific binding.

working definition of inhibitory activity; namely, the inhibitor enhances K_D^{app} without altering the concentration of hormone binding sites.

Estimation of molecular size of inhibitor

The next series of experiments was undertaken to elucidate the molecular nature of the inhibitory substance isolated as the 50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate. Table 2 summarizes the inhibitory influences of the 50–70%-satd. preparation for many experiments and shows the inhibitor to be non-diffusible. In addition, elution of this fraction with TDG 30% from 10 ml Sephadex G-25 mini-columns showed the inhibitory substance to be confined to the void volume (results not shown).

The gel filtration characteristics of the 50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction were examined in greater detail by using a calibrated column containing Sephadex G-75 equilibrated and eluted with incubation buffer (TDG 30%). The elution profile from a

Table 2. Effects of various inhibitory preparations on progesterone-receptor binding

Summary data from several experiments are presented. Aliquots (100 μl) of each inhibitor preparation were incubated with 100 μl of receptor from the same pool of cytosol. Binding constants were determined by exchange assay under equilibrium conditions. Values are means $\pm$ S.E.M. The number of experiments is given in parentheses. Analysis of variance indicated that K_D^{app} is altered by inhibitor preparations ($P < 0.005$); however, the number of binding sites was not altered by any of the preparations ($P > 0.05$). The receptor was prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation (0–45%-satd. fraction). Protein concentration was 0.12 ± 0.01 mg/tube. Values differ from control, * $P < 0.05$; ** $P < 0.01$. Statistical analysis was by the Student–Newman–Keuls multiple range test. Inhibitor was prepared as the 50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction (0.19 ± 0.017 mg/tube). A 1.0 ml aliquot of inhibitor fraction was dialysed against 100 vol. of Tris buffer, pH 7.8 at 4°C , for 24 h in a dialysis membrane which excluded molecules larger than mol.wt. 12000–14000.

Preparation	K_D^{app} (nM)	B_{max} (pmol/mg)
Receptor (19)	2.50 ± 0.18	0.319 ± 0.028
+ Inhibitor (19)	$7.60 \pm 0.65^{**}$	0.419 ± 0.039
+ Dialysed (4) inhibitor	$6.36 \pm 0.36^*$	0.376 ± 0.054

representative experiment is presented in Fig. 6. Two fractions exhibited significant inhibitory activity and were estimated to contain substances with a Stokes radius of 2.55–3.15 nm. The average mid-point of the active fractions was 2.88 ± 0.12 nm. All five experiments yielded inhibitory activity confined to these fractions.

Influence of sulphydryl reagents on inhibitory activity

The role of functional sulphydryl groups for inhibitor activity was investigated with HgCl_2 and *N*-ethylmaleimide substances which preferentially denature disulphide bridges (Brown & Edwards, 1969; Liu, 1977) and a sulphydryl alkylating agent, respectively. Exposure of the 50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction to 2.4 mM- HgCl_2 for 3 h destroyed inhibitory activity. However, similar treatment of an aliquot with 2.2 mM-*N*-ethylmaleimide had no influence on inhibitor activity (Fig. 7). This concentration of *N*-ethylmaleimide is sufficient to prevent thiol-dependent progesterone–receptor binding (Ogle, 1980).

Association rate studies

This report shows that an inhibitor is present which describes the affinity of receptor for pro-

Fig. 6. Elution profile of the inhibitory [50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$] fraction from trophoblast cytosol. Fractions (0.46 ml) were collected from a 60 ml column packed with Sephadex G-75 and combined into five pools (stippled bars). Elution volumes of the following internal markers are indicated by arrows: void volume, V_0 ; ovalbumin, Ov; chymotrypsinogen A, Ch. Aliquots (100 μl) of each eluate pool were placed in glass tubes with an equal volume of receptor to determine inhibitory activity. A saturation assay was performed under equilibrium conditions with six concentrations of $[^3\text{H}]$ progesterone (0.8–22 nM). The control tubes contained 100 μl aliquots of buffer eluted from the column before V_0 . Inhibitory activity of each pool (ability to increase $K_{\text{D}}^{\text{app}}$) is depicted relative to progesterone–receptor binding without inhibitor by the height of the stippled bars with reference to the right-hand ordinate. Representative results of four experiments are shown. $\bullet$, A_{280} ; $\circ$, protein by the Lowry method.

Fig. 7. Effects of HgCl_2 and N -ethylmaleimide on inhibitor activity

Inhibitor [50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$] fraction (1.5 ml of 1.44 mg/ml) was incubated with 2.2 mM- HgCl_2 or 2.4 mM- N -ethylmaleimide for 3 h at 0°C , then dialysed against 3 changes of 100 vol. of buffer over a period of 24 h to assure quantitative removal of unreacted reagent. An aliquot of untreated 50–70%-satd. fraction was similarly dialysed against buffer. Aliquots of non-diffusible material were incubated with freshly prepared receptor (0–45%-satd. fraction) to assay inhibitory activity. $\circ$, receptor (0–45%-satd. fraction); $\blacktriangle$, receptor + 50–70%-satd. fraction; $\bullet$, receptor + HgCl_2 treated 50–70% satd. fraction; $\triangle$, receptor + N -ethylmaleimide-treated 50–70%-satd. fraction. The open and filled circles produced superimposable regression lines.

gestosterone. It is of interest to determine whether the effect is mediated by impaired association of ligand and receptor or by an enhanced rate of dissociation. It was initially presumed that second-order kinetics (bimolecular reaction) should adequately describe the reaction. The association reaction was therefore examined as a second-order rate function:

$$\frac{2.3}{([P]_0 - [R]_0)} (\log [P]_t / [R]_t) = k_{+1}(t) + C \quad (1)$$

where $[P]_0$ and $[P]_t$ are the concentration of free $[^3\text{H}]$ progesterone at zero time and at time t , respectively; $[R]_0$ and $[R]_t$ are the concentrations of unbound receptor sites at zero time and at time t , respectively. Association rate studies were performed with trophoblast progesterone cytosol receptor. The association rate remained linear for at least 14 min. The contribution of nonspecific binding to the association reaction was studied by incubating receptor with $[^3\text{H}]$ progesterone and a 100-fold molar excess of either unlabelled progesterone or unlabelled cortisol. Unlabelled progesterone successfully competed for $[^3\text{H}]$ progesterone binding sites whereas cortisol was unable to do so, signifying the specificity of the reaction (results not shown).

The association rate for receptor in whole cytosol was highly dependent upon the concentration of cytosol. The association rate constant increased with

dilution of the cytosol, which is probably a result of a shift in the equilibrium between receptor and the endogenous inhibitory macromolecule. However, the rate of association became independent of protein concentration when receptor was partially purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation. A concentration range of 0.6–2.6 nM binding sites of partially purified receptor exhibited an association rate of $(1.98 \pm 0.58) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $n = 19$. The rate of ligand–receptor complex formation was also independent of the ratio of the concentration of reactants

$([\text{P}]_0/[\text{Rp}]_0)$ over a range of 1.6 to 10. However, the association rate was markedly reduced by the inhibitor (Fig. 8 and Table 3).

Dissociation rate kinetics

Theoretical considerations dictate that the dissociation of ligand from receptor follow first-order kinetics:

$$[\text{P-Rp}]_t = [\text{P-Rp}]_0 e^{-kt} \quad (2)$$

where k is the dissociation rate constant, $[\text{P-Rp}]_0$ and $[\text{P-Rp}]_t$ are the concentrations of bound receptor at time zero and time t , respectively. The molar concentration of ligand–receptor remaining as a function of time of dissociation should yield a negative exponential curve, the initial rate of which is the dissociation rate constant (k_{-1}). However, deviation from a single exponential function could be attributable to the presence of unsaturable non-specific binding sites (Rodbard, 1973):

$$[\text{P-Rp}]_t = [\text{P-Rp}]_0 e^{-kt} + N \quad (3)$$

where N is the non-specific component and may be resolved as the horizontal asymptote of the arithmetic plot (Fig. 9).

Additional binding moieties could contribute to the dissociation curve. This possibility was explored by fitting the data to multi-term exponential equations, as would be applicable if several rate constants were encountered simultaneously (Rodbard, 1973). The data were fit to these models by the iterative method of Marquardt. The relative goodness of fit was compared quantitatively by the F -test (Sprague *et al.*, 1980).

Eqn. (2) did not fit the data and resulted in a large mean square residual error term; however, utilization of eqn. (3) significantly reduced the error term ($P < 0.05$). Hypothesizing additional binding moieties gave no further reduction in the mean square residual error; thus, there was no evidence to suggest the presence of a secondary binder in this system. The deviation from a single exponential function could be fully accounted for by the presence of functionally unsaturable nonspecific binding sites (eqn. 3). These results indicated that the binding of

Fig. 8. Effect of inhibitor on the association rate of progesterone and receptor at 0°C

Receptor and inhibitor were prepared as the 0–45% and 50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction respectively. The associate rate constant (k_{+1}) was calculated from the slope of the plot. Representative results of ten experiments are shown. ●, Receptor (0–45%-satd. fraction), $k_{+1} = 1.037 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; ○, receptor + inhibitor (50–70%-satd. fraction), $k_{+1} = 0.505 \times 10^4 \cdot \text{s}^{-1}$.

Table 3. Association and dissociation rate constants for progesterone binding to trophoblast cytosol receptor. Values are means $\pm$ S.E.M. with the number of experiments in parentheses. Receptor and inhibitor were prepared from the same pool of cytosol as the 0–45%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction (2–3 mg of protein) and 50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction (1–2 mg of protein), respectively. *Mean value differs from control group, $P < 0.01$ by paired t -test.

Preparation	$10^{-4} \times k_{+1} (\text{M}^{-1} \cdot \text{s}^{-1})$ (5)	$10^5 \times k_{-1} (\text{s}^{-1})$ (5)	$k_{-1}/k_{+1} (\text{nM})$	$K_D (\text{nM})$ at equilibrium (19)
Receptor	1.28 ± 0.22	3.20 ± 0.61	2.5	2.5 ± 0.2
Receptor + inhibitor	$0.36 \pm 0.06^*$	2.74 ± 0.36	7.6	$7.6 \pm 0.7^*$

Fig. 9. Effect of inhibitor on dissociation rate (k_{-1}) for the trophoblast progesterone-receptor complex. Receptor [0–45%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction], 0.7 mg of protein/ml] $\pm$ inhibitor (50–70%-satd. fraction, 0.4 mg of protein/ml) was incubated for 18 h at 0°C with a saturating concentration of [^3H]progesterone (15.8 nM). After equilibration, free steroid was removed by dextran/charcoal, a 100-fold molar excess of unlabelled progesterone was added and the rate of decrease of bound [^3H]progesterone was monitored (see the Material and methods section). The inset shows data corrected for nonspecific binding (N). Representative results for eight experiments are shown. ●, Receptor, $k_{-1} = 4.9 \times 10^{-5} \text{ s}^{-1}$; ○, receptor + inhibitor, $k_{-1} = 4.6 \times 10^{-5} \text{ s}^{-1}$.

progesterone with receptor could be resolved into a single specific high affinity system and a non-specific, nonsaturable component (Fig. 9). The values of the dissociation rate constant were similar for whole cytosol and partially purified receptor and were independent of protein concentration. The inhibitor had no effect on the rate of dissociation.

Table 3 summarizes the influence of inhibitor on the association and dissociation rate constants for receptor. The final column of the Table presents the mean value of K_D determined under equilibrium conditions which, notably, is in agreement with K_D estimated from the ratio of the rate constants.

Fig. 10. Arrhenius plot showing the effect of inhibitor on the energy of activation (E_a) for progesterone-receptor complex formation. E_a was calculated as the slope of the plot. Representative results for five experiments are shown. ●, Receptor [0–45%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction], $E_a = 44.5 \text{ kJ/mol}$; ○, receptor + inhibitor (50–70%-satd. fraction), $E_a = 59.8 \text{ kJ/mol}$.

Table 4. Influence of inhibitor on the energy of activation (E_a) for the progesterone-receptor complex. Values are means $\pm$ S.E.M.; number of experiments are given in parentheses. Receptor and inhibitor were prepared as the 0–45%- and 50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$ fractions, respectively. *Mean differs from control value, $P < 0.05$, by Students' t -test.

Preparation	E_a (kJ/mol) for	
	Association	Dissociation
Receptor	33.5 ± 4.0 (8)	176.9 ± 21.9 (4)
Receptor + inhibitor	61.5 ± 8.2 (5)*	177.6 ± 6.3 (3)

Effects of temperature on progesterone-receptor kinetics and influence of inhibitor

The preceding findings showed that the association and dissociation of progesterone and receptor were in accordance with the theoretical expectations of a simple bimolecular system following the laws of mass action. Introduction of the inhibitory substance to this scheme alters only the equilibrium of this system by slowing the rate of association. However, rate constants are, them-

selves, a function of non-equilibrium thermodynamics related by the Arrhenius equation:

$$k = Ae^{-E_a/RT} \quad (4)$$

where k is the rate constant, A is a frequency factor for fruitful interaction between reactants, E_a is the energy of activation, R is the gas constant, and T is the absolute temperature. Thus, it was of interest to examine thermodynamic aspects of progesterone-receptor-inhibitor interaction. The rate of association and dissociation of complex was studied at 0, 15 and 30°C in the presence and absence of the inhibitor and the results were analysed by using the Arrhenius plot. Previous studies have shown that the receptor is thermolabile (Ogle, 1980) and rapidly denatured at 30°C. However, preliminary experiments showed that the addition of 10 mM-molybdate to the TDG-30% buffer prevented denaturation of receptor, at least during the 14 min period of reaction. These early studies also revealed that molybdate had no influence on binding parameters under these conditions.

The Arrhenius plots were linear in the presence and absence of inhibitor, which indicates that the same rate-limiting step occurs at all temperatures. However, the energy of activation (E_a) for progesterone-receptor binding was increased almost 100% by the inhibitor ($P < 0.05$). A representative experiment is shown in Fig. 10. The inhibitor had no influence on E_a of the dissociation reaction. These findings are summarized in Table 4.

Discussion

These studies indicate the presence of a specific inhibitor within the cytoplasm of rat trophoblast. The inhibitor decreased the affinity of progesterone for receptor. This substance could be isolated from receptor by heating cytosol to 30°C for 30 min (receptor proteins were denatured) or as the 50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate. The kinetic behaviour of these inhibitor preparations was invariable whether tested with whole cytosol or partially purified receptor [0–45%-satd. $(\text{NH}_4)_2\text{SO}_4$]. The purified receptor showed an K_D^{app} of $2.5 \pm 0.2 \text{ nM}$ with a sedimentation coefficient of about 8S. Incubation of receptor with the inhibitor fraction (50–70%-satd.) increased K_D^{app} 26-fold per mg of protein, but the concentration of receptor binding sites was not influenced (shown by sucrose gradient analysis and by mass action behaviour at equilibrium, see Fig. 5 and Table 2).

Physicochemical studies of the inhibitor, prepared as the 50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate, showed that it was non-diffusible, excluded from Sephadex G-25, not adsorbed to dextran/charcoal, and stable at 35°C for 30 min but irreversibly denatured when warmed to 70°C for 30 min. A

specific tertiary conformation appears necessary for biological activity of the inhibitor since activity was destroyed by HgCl_2 .

The molecular size of the inhibitory substance was estimated by determination of the elution volume of active fractions of the 50–70%-satd. $(\text{NH}_4)_2\text{SO}_4$ pellet from a calibrated Sephadex G-75 column. Unfortunately, 1.4 ml of eluate were required for assay of inhibitor activity and thus, active fractions could not be subdivided further. Therefore estimates of the effective Stokes' radius cover a broad range from 2.55 to 3.15 nm. The centre of the inhibitor-active fractions averaged $2.88 \pm 0.12 \text{ nm}$. Although many physicochemical properties of this protein-like substance are unknown, it is not a serum albumin since the latter elutes from this column with an effective Stokes' radius of 3.5 nm.

The rate of association of progesterone with the trophoblast receptor followed second-order kinetics; however, it was 10-fold slower than that reported for some oestrogen-receptor systems (Korach & Muldoon, 1974; Sanborn *et al.*, 1971), the progesterone receptor system in chick oviduct (Hansen *et al.*, 1976) and uterus of rat and mouse (Feil *et al.*, 1972). The inhibitor caused a 3–4-fold decrease in the rate of association. The process of dissociation described first-order kinetics and showed that the cytosol was free from secondary dissociating species. The rate of dissociation of complex was equal to that observed in oestradiol-receptor systems (Korach & Muldoon, 1974; Sanborn *et al.*, 1971). The inhibitor had no influence on the dissociation rate of the ligand-receptor complex. Thus, the 10-fold difference in affinity of progesterone for trophoblast receptor under equilibrium conditions compared with oestradiol-receptor systems is fully accounted for by slow association kinetics.

That the affinity of this progesterone-receptor system estimated both as the ratio of the rate constants and under equilibrium conditions are in agreement strongly suggests that the pathway of binding obeys the simple equilibrium relationship:

The same holds true when the inhibitory macromolecule is introduced into the scheme. However, the rate of association of ligand and receptor is impaired, which shifts the equilibrium to the left. This behaviour is consistent with an equilibrium scheme wherein receptor binding sites for progesterone and inhibitor may be distinct but overlapping or inhibitor may bind to a distinct inhibitor site which distorts the ligand binding site slowing the association rate. However, once progesterone binds receptor either the inhibitor can no longer bind or its binding fails to alter an occupied site and hence dissociation proceeds unhindered.

That a conformational change in receptor actually occurs as a result of the inhibitor is supported by the observed increase in E_a for the association reaction. The change in E_a is probably due to a more unfavourable conformation of the receptor binding site such that nearly twice the energy is required for proper alignment of the receptor binding site with ligand.

Linearity of the Arrhenius plots for the association and dissociation reactions is consistent with the equilibrium represented by eqn. (5), even in the presence of inhibitor, wherein each reaction is limited by a single rate constant. No other reaction steps become significant at other temperatures. In this way, then, the inhibitor is a potential regulator of progesterone action. However, it would not be expected to alter the biological potency of complexed receptor since this property is a function of the dissociation rate. Of course, the physiological significance of the inhibitor lies in the mechanisms which control its activity and the manner in which the inhibitor contributes to survival of the species.

These aspects of ligand-protein interaction have not yet received a great deal of attention. However, previous experiments (Ogle, 1981) have shown that inhibitor activity in trophoblast cytosol is greatest on days 9 and 12 of pregnancy but decline thereafter. By day 18 inhibitory activity is no longer detected. This period is also characterized by a sharp decrease in the concentration of receptor. Thus, it appears that the levels of the rat trophoblast progesterone receptor and inhibitor fluctuate and may play regulatory roles in maintenance of pregnancy.

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