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Resolution of High and Low Affinity Progesterone Receptors from Human Breast Carcinoma T47D Cells*

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Each of four independent experimental approaches showed that human breast carcinoma T47D cells contain both high and low affinity progesterone receptors. (i) Equilibrium-specific [^{3}H]progesterone binding to adherent cultured cells revealed dissociation constants (K_d) of 1.5 and 60 nM and 0.33 and 2.4 × 10^6 sites/cell, respectively. Both the high and low affinity receptors were specific for progestins as demonstrated by steroid binding competition studies conducted at 5 and 50 nM [^{3}H]progesterone. (ii) Equilibrium [^{3}H]progesterone binding to the resolved soluble and particulate fractions from a cell homogenate sedimented at 40,000 × g·min revealed K_d = 1.4 nM high affinity binding sites exclusively in the supernatant fraction and K_d = 24 nM low affinity sites exclusively in the particulate fraction. Extraction of the particulate fraction with a high ionic strength buffer solubilized the low affinity receptors stoichiometrically; but once solubilized, they displayed K_d = 2.4 nM high affinity progesterone binding. Characterizations of ^{3}H-ligand bound specifically to progesterone receptors in intact cells or resolved subcellular fractions revealed no [^{3}H]progesterone metabolites that could account for the low affinity binding. (iii) Calculations based on the rate constants of [^{3}H] progesterone association with or dissociation from adherent cells revealed the same dissociation constants for both high and low affinity binding as those determined by equilibrium measurements. (iv) Nonionic detergent extraction of cells incubated with a wide range of [^{3}H]progesterone concentrations revealed high affinity progesterone binding to receptors in the detergent-soluble fraction and low affinity binding associated primarily with the particulate residue, consistent with the data on equilibrium progesterone binding to resolved cell homogenate fractions. The rate of extraction of the high affinity receptor-progesterone complex with nonionic detergent ($t_{½}$ = 1 min at 0 °C) equaled the rate of extraction of a representative lysosomal enzyme, β-acetylglucosaminidase.

A considerable body of information has favored the view that steroid receptors, including progesterone receptors, are cytoplasmic proteins with a capacity to acquire a nuclear DNA-binding form upon interaction with ligand in intact cells (for reviews, see Refs. 1–3). Incubation of viable suspended breast carcinoma cells with progesterone is reported to lead first to translocation of cytoplasmic progesterone receptors to the nuclear fraction and then to loss of nuclear progesterone binding activity (4). Ligand-dependent, cytoplasmic to nuclear translocation of steroid receptors has been challenged based on results of immunocytochemical localization and cytochalasin-induced enucleation studies showing that estrogen receptors are predominantly nuclear in cells not exposed to ligand (5, 6). Similarly, Perrot-Applanat *et al.* (7) employed immunocytochemistry to show that rabbit uterine progesterone receptors are primarily in the nucleus with a small portion in the cytoplasm.

Studies with purified chick oviduct progesterone receptor have revealed two species or "subunits" with molecular sizes of 79 and 108 kDa (8–10). Each binds progesterone with a dissociation constant of approximately 2 nM, and both have similar ligand binding specificities (8, 12). Recently, Maggi *et al.* (12) reported evidence for high and low affinity classes of binding in purified preparations of chick oviduct progesterone receptor that lacked specific binding activity for other steroids. The dissociation constant for the high affinity class in this preparation was equivalent to that reported previously (8). High and low affinity classes of progesterone binding have been observed in extracts of MCF-7 cells, a human breast carcinoma line. Steroid binding competition studies indicated that the glucocorticoid receptor accounted for the lower affinity binding (13). Other investigators (14) have observed only a single class of progesterone-binding sites in MCF-7 cell extracts. A single class of progesterone-binding sites with a dissociation constant of approximately 2 nM has been demonstrated in extracts derived from human breast carcinoma T47D cells that contain high levels of progesterone receptor, but negligible levels of glucocorticoid or estradiol receptors (15, 16).

The chick oviduct progesterone receptors are high affinity substrates *in vitro* for stoichiometric phosphorylation by the epidermal growth factor and insulin receptor protein-tyrosine kinases (17, 18). In preparation for studies to test for physiological relevance of these observations, we first characterized the binding of progesterone to its receptor in intact T47D cells. We report here observations that adherent T47D cells contain both high and low affinity classes of equilibrium progesterone-binding sites. The high affinity sites are those associating with the cytosolic fraction upon homogenization, and the low affinity sites are those sedimenting with the particulate fraction of a cell homogenate.

EXPERIMENTAL PROCEDURES

Materials—[1,2,6,7-^{3}H]Progesterone (91 Ci/mmol) was obtained from Amersham Corp. [^{3}H]R5020 ([17α-*methyl*-^{3}H]17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione; promegestone) and unlabeled R5020 were from Du Pont-New England Nuclear. Unlabeled proges-

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terone, all other steroids, leupeptin, dithiothreitol, EDTA, EGTA,[1] and phenylmethylsulfonyl fluoride were purchased from Sigma. Unless otherwise stated, all other chemicals were of reagent-grade.

Cells and Cell Culture—Human breast carcinoma T47D cells, obtained from Marc Lippman (National Cancer Institute, Bethesda, MD), were maintained in 75-cm^2 polystyrene culture flasks in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) containing 5% fetal bovine serum (GIBCO) and 10 μg/ml gentamycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were grown to near confluency and passed (1:10 split) once a week with a change in culture medium on the third day.

Cells were grown for adherent cell binding studies in 24-well polystyrene dishes (Corning Glass Works, Corning, NY) for 3 days to about 10^5 cells/cm^2 (50% confluent). Cells were cultured only in the first and fourth row wells of the four-row 24-well dish since cells in the inner and outer wells sometimes behaved differently. For progesterone-binding experiments on suspended cells, cells were cultured in 55-cm^2 polystyrene tissue culture Petri plates (Corning Glass Works) to 50–70% confluency.

In binding experiments involving supernatant and particulate fractions from cell homogenates, T47D cells were grown with a change in the medium on the third day of culture in 800-cm^2 roller bottles in Dulbecco's modified Eagle's medium (Irvine Scientific) and Ham's F-12 (GIBCO), in a 1:1 ratio, containing 10% calf serum (M. A. Bioproducts, Walkersville, MD) and 10 μg/ml gentamycin. Roller bottles were gassed with CO_2, and the cells were harvested when approximately 80% confluent.

Preparation of Cell Suspensions—T47D cell suspensions were obtained from cells grown in either Petri plates or roller bottles. The cells were washed twice with 10 or 50 ml of Tris-buffered saline (150 mM NaCl and 10 mM Tris-HCl (pH 7.4)) per Petri dish or roller bottle, respectively. The cells were detached by incubating at 37 °C for 15 min in 5 or 15 ml Tris-saline/EDTA (25 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, and 1 mM Na_2HPO_4 (pH 7.4 at 37 °C)) per Petri dish or roller bottle, respectively. During this incubation period, the bottles were rolled continuously at 4 rpm, whereas Petri plates were incubated without shaking. At the end of the incubation in Petri plates, the cells were detached by repeatedly pipetting a stream of Tris-saline/EDTA solution across the surface of the dish.

Preparation of High Speed Cytosol and Particulate Fractions following Homogenization—Suspended cells were collected by a 4,000 × *g*·min centrifugation, washed twice with 10 cell pellet volumes of homogenization buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 40 μg/ml leupeptin, 10 mM sodium molybdate, and 10% (v/v) glycerol (pH 7.4)), and homogenized in 2 pellet volumes of ice-cold homogenization buffer using 50 strokes of a Dounce homogenizer. The cell homogenate was centrifuged at 40,000 × *g*·min, and the supernatant fraction was collected. The particulate fraction was washed once with 1 volume of homogenization buffer and centrifuged at 40,000 × *g*·min. These combined supernatant fractions were centrifuged at 6 × 10^6 × *g*·min to obtain the high speed cytosol. The 40,000 × *g*·min pellet was suspended in 3 volumes of homogenization buffer to determine progesterone binding to particulate fraction-associated receptors. The high speed cytosol was diluted 5-fold prior to binding assays.

Extraction of Particulate Fraction-associated Progesterone Receptors at High Ionic Strength—Washed pellets were suspended in 3 volumes of homogenization buffer containing 0.5 M KCl. The suspension was incubated with gentle shaking on a rotating platform at 4 °C for 60 min and then centrifuged at 40,000 × *g*·min. The supernatant fraction containing receptors solubilized from the particulate fraction was diluted 3-fold and used for progesterone binding experiments. Where indicated, 1.0 ml of the 0.5 M KCl extract containing 2.0 mg of protein was desalted at 4 °C using a 0.9 × 13-cm Sephadex G-25 column equilibrated with homogenization buffer.

Progesterone Binding to Adherent Cells—Progesterone binding to adherent cells was performed on cells grown in 24-well culture dishes. Prior to ligand binding, cells were washed twice with 2 ml of binding medium (10 mM Hepes-buffered RPMI 1640 medium (pH 7.2 at 37 °C)) per well per wash and incubated at 22 °C for 60 min in 0.5 ml of the binding medium containing 0.25–150 nM [^{3}H]progesterone. Stock solutions of progesterone were made in 95% ethanol, and the maximum ethanol concentration during binding was 0.5%. Replicate assays contained 10 μM unlabeled progesterone for determination of nonspecific binding. At the end of incubations, the 24-well dishes were placed on ice and washed three times with 2 ml of ice-cold binding medium to remove free ligand. Cells were then incubated with 300 μl of 1 M NaOH for 60 min at 60 °C, and 150 μl of the NaOH-solubilized material was neutralized with HCl for assay of radioactivity in 7 ml of aqueous counting scintillation fluid (ACS) (Amersham Corp.).

Progesterone Binding to Receptors Present in Cytosol and Salt-extracted Particulate Fractions—Aliquots of 50 μl from the high speed cytosol, particulate extract, or the desalted particulate extract were incubated at 4 °C for 16 h with 50 μl of homogenization buffer containing varying concentrations of [^{3}H]progesterone. Final [^{3}H] progesterone concentrations ranged from 0.2 to 100 nM. At each progesterone concentration, three independent determinations were performed; nonspecific binding was that remaining in the presence of 10 μM unlabeled progesterone. At the end of incubations, bound radioactivity was determined by the dextran-coated charcoal assay (19). One part (v/v) of dextran-coated charcoal suspension (0.025% dextran and 0.5% charcoal (w/v) in homogenization buffer) was added per sample. After mixing by Vortex action, the samples were incubated at 0 °C for 10 min and centrifuged at 47,000 × *g*·min. A 50-μl aliquot of the supernatant fraction was used for determination of radioactivity in 7 ml of ACS.

Progesterone Binding to Receptors Present in Particulate Fraction—Aliquots of 50 μl of pellet suspension were incubated with [^{3}H] progesterone as described for the cytosol fractions. Binding was terminated by centrifuging the samples at 47,000 × *g*·min, and the supernatant fraction was removed by aspiration. Pellets were washed twice by suspension in 0.5 ml of homogenization buffer and centrifugation at 47,000 × *g*·min. The washed pellets were extracted with 500 μl of homogenization buffer containing 0.5 M KCl for 60 min at 4 °C with gentle shaking on a rotating platform. Suspensions were then centrifuged at 47,000 × *g*·min, and a 250-μl aliquot of each supernatant fraction was quantified for radioactivity in 7 ml of ACS.

Fractionation of Bound Progesterone by Extraction of Cells with Nonionic Detergent—After equilibrium binding of [^{3}H]progesterone to adherent cells was achieved, and following the standard wash procedure to remove free ligand, cells were treated with 0.5 ml of 0.5% (w/v) Triton X-100/2-cm^2 well in 10 mM Hepes-buffered RPMI 1640 medium (pH 8.0 at 4 °C) at 0 °C for 5 min. The dishes were shaken briefly following 2 min of treatment with nonionic detergent and then again at 5 min prior to collection of the supernatant fraction. Residual solution in the wells was removed promptly by aspiration. Light microscopic examination of the cell substratum-adherent Triton X-100 residue revealed only nuclei and some fibrous material around the nuclei. This procedure for nonionic detergent extraction of adherent cells is based on the method of Brown *et al.* (20), who showed that following extraction of adherent cells with 0.5% Triton X-100, only nuclei, surrounding cytoskeleton, and cell matrix remained adherent to the culture dish. A 0.25-ml aliquot of Triton X-100-extracted material was quantified for radioactivity in 7 ml of ACS. The detergent-nonextractable material was solubilized in 300 μl of 1 M NaOH for 60 min at 60 °C, and 150 μl of this was neutralized with HCl and quantified for radioactivity in 7 ml of ACS.

β-Acetylglucosaminidase Assay—The lysosomal enzyme activity in 0.5% Triton X-100 extracts of adherent T47D cells, prepared as described above, was determined by the "Hand Assay" of Baggiolini (21).

Protein Determination—Protein content in samples was determined using a Bio-Rad protein assay kit and Cohn fraction V bovine serum albumin (Sigma) as the standard.

RESULTS[2]

Equilibrium Progesterone Binding to Cells Reveals Two Classes of Affinity—Adherent T47D cells were incubated with a wide range of progesterone concentrations (0.25–150 nM) to achieve equilibrium binding which was approached after a 60-min incubation (Fig. 1*C*). Total bound, specifically bound, and nonspecifically bound [^{3}H]progesterone as functions of

[1] The abbreviations used are: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ACS, aqueous counting scintillation fluid.

[2] Portions of this paper (including part of "Results," Fig. 7, and Tables VII–IX) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

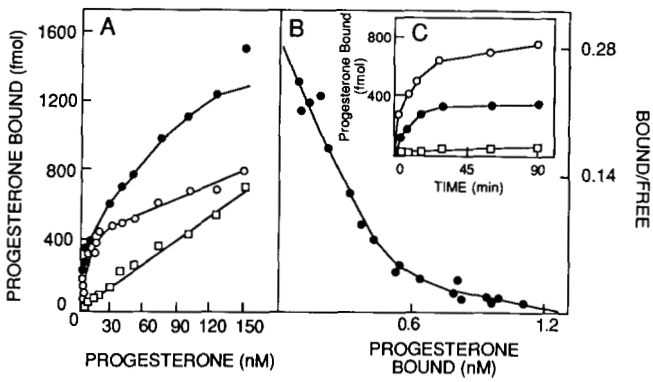

FIG. 1. **Equilibrium progesterone-specific binding to substratum-adherent human breast carcinoma T47D cells.** Adherent cells were incubated *in situ* in 0.5 ml of binding medium containing 0.25–150 nM [^{3}H]progesterone for 60 min at 23 °C. They were then washed free of ligand at 4 °C in binding medium and solubilized in 1 M NaOH for determination of radioactivity as described under "Experimental Procedures." Nonspecific binding occurring in the presence of 10 μM unlabeled progesterone was deducted from each point. *A*, total (●), specifically (○), and nonspecifically (□) bound progesterone/2 × 10^5 cells; *B*, Scatchard plot of the data shown in *A*, *C*, time courses for binding with [^{3}H]progesterone present at 0.5 (□), 20 (●), and 150 (○) nM. Each point represents the average of duplicate determinations.

ligand concentration are shown in Fig. 1*A*. In the experiment shown, nonspecific binding was determined using 10 μM unlabeled progesterone to compete for specific binding of labeled progesterone to its cellular receptors. This concentration is approximately 170-fold the dissociation constant calculated for the low affinity sites (discussed below) and 60-fold greater than the highest concentration of labeled ligand employed in the binding studies. Nonspecific binding also was determined from total binding data independently using the LIGAND program to treat nonspecific binding as a parameter subject to error and which is fitted simultaneously with other parameters (22). Nonspecific binding determined by both these methods was in close agreement, was linear as a function of ligand concentration, and was less than 50% of total binding at the highest [^{3}H]progesterone concentration used (see Fig. 1*A*).

The specific binding data from Fig. 1*A* are presented in a Scatchard representation in Fig. 1*B*. The curve has upward concavity, indicating the presence of two classes of progesterone-binding sites. When analyzed with the LIGAND program described by Munson and Rodbard (22), a two-class progesterone-binding site model was statistically far superior to a one-class binding site model as determined by an *F*-ratio test (22) (level of significance, $p < 0.001$). Analysis of [^{3}H]progesterone bound at equilibrium to T47D cells also was done where nonspecific binding was established from the raw data using LIGAND for this purpose as described by Munson and Rodbard (22). With nonspecific binding determined computationally, the data also fitted the two-site model better than the one-site model (*F*-ratio test, $p < 0.01$) and yielded approximately the same K_d and receptor number values as those obtained by analysis of data where nonspecific binding was determined experimentally (Table I).

The high affinity progesterone-binding sites in T47D cells had a dissociation constant of approximately 1.5 nM, in agreement with values reported previously for T47D cells, human breast carcinoma MCF-7 cells, and human myometrium or chick oviduct cytosol fractions (Refs. 15, 13, 1, and 11, respectively). The previously undescribed low affinity sites bound progesterone with K_d = 60 nM and comprised most of the saturable cellular progesterone binding (Table I).

TABLE I

Progesterone binding by adherent T47D cells

Analyses of data from the experiment described for Fig. 1 and from two independent experiments were conducted using the LIGAND program of Munson and Rodbard (22). The K_d and receptor number values in A were based on experimentally determined nonspecific binding; whereas in B, the values were obtained with LIGAND using a strictly computational approach for determining nonspecific binding. Values are means from the three independent experiments ± S.D.

	K_d	Receptor number
	nM	*×10⁶ sites/cell*
A. Low affinity	60.0 ± 8.0	2.40 ± 1.0
High affinity	1.5 ± 0.5	0.33 ± 0.15
B. Low affinity	55.0 ± 10	2.30 ± 0.8
High affinity	1.5 ± 0.4	0.37 ± 0.20

Tests for Relationships of [^{3}H]Progesterone Metabolism to Detection of Both High and Low Affinity Progesterone Binding—The detection of saturable low affinity progesterone-binding sites was not related to the formation of [^{3}H]progesterone metabolites, nor was it related to depletion of the hormone by cell metabolism (see Miniprint). Moreover, the two classes of receptors can be resolved by cell homogenization and subcellular fractionation. There was no significant metabolism of [^{3}H]progesterone by these resolved fractions under conditions where their binding properties were determined.

Progesterone Binding to T47D Cells and Progesterone Receptor Subcellular Distribution—Incubation of intact T47D cells at 37 °C with 0.01–1.0 μM progesterone is reported to induce translocation of progesterone receptors from the cytosol (soluble fraction) to the nucleus (particulate fraction) (4). The redistribution of progesterone receptors was reported to depend upon progesterone concentration, with a near maximal and rapid translocation occurring, within 5 min, at 100 nM progesterone (4). However, at 24 °C, progesterone-induced translocation of receptors was slow (4). We re-examined this phenomenon under precisely the conditions described by Mockus and Horwitz (Table III in Ref. 4) to determine if the low affinity sites arose by subcellular redistribution of progesterone receptors following exposure of cells to progesterone. T47D cell suspensions were incubated at 37 °C for 2–60 min with 100 nM [^{3}H]progesterone. The cells were homogenized, and the soluble and particulate fractions were separated as described by Mockus and Horwitz (Table III in Ref. 4). Specific [^{3}H]progesterone binding in the supernatant fraction achieved equilibrium rapidly, whereas binding in the particulate fraction approached equilibrium more slowly (Table II). There was no evidence for translocation of progesterone receptors from the soluble to particulate fraction and no progressive loss of progesterone binding from either fraction (Table II). These same results were obtained in two additional independent experiments. We observed that under published extraction conditions, *i.e.* 30% glycerol present, extraction of specifically bound [^{3}H]progesterone from the particulate fraction was incomplete. Stoichiometric extraction was achieved when the glycerol concentration in the extraction buffer was reduced to 10% (see Table IV).

Tests for progesterone receptor translocation also were made by comparing progesterone receptor distribution in the soluble and particulate fractions of progesterone-treated and -untreated cells. Suspended T47D cells were incubated at 22 °C in the presence or absence of 50 nM [^{3}H]progesterone, a concentration reported to cause effective translocation of progesterone receptors (4). This was followed by resolution of

TABLE II

Specific progesterone binding to suspended cells at 37 °C

Suspended T47D cells (1×10^6) were incubated in 1.5 ml polypropylene tubes at 37 °C for 60 min in 0.5 ml of binding medium containing no serum and then in binding medium containing 100 nM [^{3}H]progesterone for 2, 7, or 60 min at 37 °C. Replicate incubations were performed with 10 μM unlabeled progesterone to determine nonspecific binding. Samples were chilled at 0 °C for 1.0 min, washed, and homogenized in buffer containing 5 mM sodium phosphate, 10 mM thioglycerol, 10% glycerol (pH 7.4 at 0 °C) and then centrifuged to separate the particulate and supernatant fractions. The particulate fraction was extracted with a solution of 10 mM Tris-HCl (pH 8.5 at 4 °C), 1.5 mM EDTA, 30% glycerol, and 0.6 M KCl following the procedure of Mockus and Horwitz (Table III in Ref. 4). Aliquots of supernatant and KCl extract of particulate fraction and KCl-extracted pellet (the latter having been solubilized with 1 M NaOH for 60 min at 60 °C and then neutralized with HCl) were quantified for radioactivity in ACS (see "Experimental Procedures"). Values are means of three independent incubations ± S.D.

Incubation time	Specific [^{3}H]progesterone binding			
	Particulate fraction		Supernatant fraction	Total
	KCl-extractable	KCl-extracted pellet		
min	*receptor no. × 10^3 sites/cell*			
2	346 ± 20	242 ± 15	501 ± 71	1090 ± 75
7	415 ± 19	308 ± 83	539 ± 77	1263 ± 154
60	698 ± 94	996 ± 160	715 ± 80	2410 ± 310

TABLE III

Influence of cellular progestin treatment on receptor distribution in the supernatant and particulate fractions of T47D cell homogenate

T47D cells were suspended in homogenization buffer at 10^7 cells/ml. In 2-ml aliquots, they were incubated at 22 °C for 60 min with no progestins or with 50 nM [^{3}H]progesterone or 50 nM [^{3}H]R5020. The cells were chilled and homogenized in the same incubation mixture at 0 °C. The cell homogenates were centrifuged at 40,000 × *g*·min, and the supernatant and pellet fractions were separated. Supernatant fraction of cells preincubated with ^{3}H-labeled progestins were treated at 0 °C with dextran-coated charcoal to determine receptor-bound hormone, whereas the supernatant fraction of progesterone-untreated cells was first incubated at 22 °C for 60 min with 50 nM [^{3}H] progesterone or 50 nM [^{3}H]R5020 and then treated with dextran-coated charcoal (see "Experimental Procedures"). The pellet fractions from cells not treated with progestins were suspended in 1.0 ml of homogenization buffer and incubated at 22 °C for 60 min with 50 nM [^{3}H]progesterone or 50 nM [^{3}H]R5020. Following this, pellet fractions from both progestin-treated and -untreated cells were washed at 0 °C with homogenization buffer, solubilized in 1 M NaOH, and quantified for radioactivity in ACS. Nonspecific binding was determined from replicate samples incubated concurrently with 50 nM labeled hormone ± 5 μM unlabeled hormone. Values are means ± S.D. from three independent determinations.

	Additions during preincubation	Fraction		Total
		Cytosol	Particulate	
		receptor no. × 10^6 sites/cell		
Exp. 1	None	1.0 ± 0.1	1.8 ± 0.2	2.8 ± 0.3
	50 nM [^{3}H]progesterone	1.1 ± 0.1	1.6 ± 0.3	2.7 ± 0.4
Exp. 2	None	0.24 ± 0.05	1.4 ± 0.2	1.7 ± 0.2
	50 nM [^{3}H]R5020	0.26 ± 0.04	1.3 ± 0.5	1.6 ± 0.5

the soluble and particulate fractions for assay of [^{3}H]progesterone binding in these fractions from progesterone-treated and -untreated cells. The number of progesterone receptors detected in the soluble and particulate fractions was the same in both progesterone-treated and -untreated cells (Table III), and prior treatment of cells with [^{3}H]progesterone did not change the total receptor number. Similar results were obtained using [^{3}H]R5020, a synthetic progestin. These data demonstrate no receptor redistribution from the soluble to particulate fraction or loss of receptor active in progesterone binding as a result of exposing cells to progesterone under conditions employed in our binding experiments.

Resolution of High from Low Affinity Progesterone Receptors by Subcellular Fractionation—Equilibrium progesterone binding to the soluble or particulate fractions from T47D cell homogenates was performed to determine the affinity of progesterone for receptors in the two fractions. Supernatant and particulate fractions, prepared from cells not exposed to progesterone as described under "Experimental Procedures," were incubated with [^{3}H]progesterone ranging from 0.2 to 100 nM; and specific progesterone binding was determined using the LIGAND program (22). The soluble fraction displayed a single class of specific binding sites with K_d = 1.4 nM. This value was the same as that of the high affinity class of binding observed in intact cells (Fig. 2*A* and Table IV). In contrast, the particulate fraction was characterized by a single class of progesterone-binding sites with K_d = 24 nM, resembling the

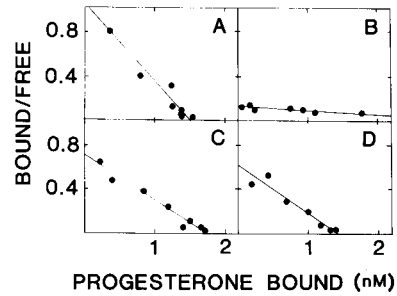

FIG. 2. **Homogenization and centrifugation separate high from low affinity progesterone receptors; extraction of the particulate fraction with high ionic strength solution converts low affinity particulate progesterone receptors to soluble high affinity receptors.** T47D cells grown in roller bottles (80% confluent) were washed, suspended, and then homogenized; and the supernatant fraction was separated from the particulate residue (see "Experimental Procedures"). The particulate fraction was divided into two portions. One was suspended gently in homogenization buffer and used directly for progesterone binding. The other was suspended in homogenization buffer containing 0.5 M KCl, incubated, and then centrifuged to yield the high ionic strength extract. A portion of that extract was desalted by elution through a Sephadex G-25 column pre-equilibrated with homogenization buffer. Equilibrium-specific [^{3}H] progesterone binding to receptors in these fractions was determined computationally as described under "Experimental Procedures" and is presented as Scatchard representation. *A*, specific binding in the high speed supernatant fraction; *B*, specific binding to receptor present in the particulate fraction; *C*, specific binding in the high ionic strength extract of the particulate fraction in the presence of 0.25 M KCl (no specific binding was detected in the salt-extracted residue); *D*, specific binding in the desalted particulate extract.

TABLE IV

Analysis of specific progesterone binding to supernatant, particulate, and particulate extract fractions

Values for K_d and receptor number were computed from the data obtained from the experiment described for Fig. 2 and two independent analyses using the LIGAND program of Munson and Rodbard (22). Values are means from these three determinations ± S.D.

Fraction	Total protein	Receptors	K_d
	mg	*pmol*	*nM*
Supernatant	1.0	6.0 ± 0.4	1.4 ± 0.2
Particulate	4.0	11.6 ± 0.3	24.0 ± 0.3
High ionic strength particulate extract	2.0	10.3 ± 0.4	2.4 ± 0.3
Desalted particulate extract	2.1	8.4 ± 0.4	2.3 ± 0.4
Salt-extracted particulate residue	2.0	<2.0	ND[a]

[a] ND, not detectable due to high nonspecific and low levels of binding.

low affinity binding sites observed in intact cells (Fig. 2*B* and Table IV).

Extraction at High Ionic Strength Converts Particulate Low Affinity Receptors to High Affinity Receptors—Low affinity progesterone receptors localized in the particulate fraction may be either a distinct class of progesterone receptors or a unique property of particulate fraction-associated high affinity receptors. We tested these possibilities by analyzing progesterone binding to particulate fraction-derived receptors solubilized in 0.5 M KCl. These bound progesterone with a dissociation constant comparable to that of high affinity receptors present in the cell homogenate supernatant fraction (Fig. 2*C* and Table IV). The receptor number in the 0.5 M KCl extract of the particulate fraction was the same as that of the unextracted particulate fraction. Extraction of progesterone binding activity from the particulate fraction was nearly quantitative. A negligible, near background level of progesterone binding activity remained in the salt-extracted particulate residue (Table IV). These data demonstrate that treatment of the particulate fraction with 0.5 M KCl extracted the low affinity progesterone receptors stoichiometrically, with their conversion to a high affinity form (Fig. 2*C* and Table IV).

The KCl extract of the particulate fraction was eluted through a Sephadex G-25 column equilibrated at low ionic strength to remove salt, and the eluate was analyzed for progesterone binding. Again, a single class of high affinity binding was observed (Fig. 2*D* and Table IV). Progesterone binding to receptors present in the supernatant fraction of cell homogenates gave dissociation constants of 1.4 ± 0.2, 1.9 ± 0.3, and 3.3 ± 1.0 nM in the presence of 0, 0.25, and 0.5 M KCl, respectively. Thus, the slightly higher value of the dissociation constant obtained with receptors extracted from the particulate fraction at high ionic strength as compared with receptors in the supernatant fraction of a cell homogenate may be an effect of salt.

Preparations of partially purified chick oviduct progesterone receptor are reported to display high and low affinity progesterone-binding sites (12). The low affinity progesterone binding with the partially purified receptor preparation was inactivated by incubation with bovine intestinal alkaline phosphatase; moreover, 10 nM glucose 1-phosphate, an alkaline phosphatase inhibitor, prevented this action of intestinal alkaline phosphatase (12). This suggested that low affinity binding by chick oviduct progesterone receptors was due to a phosphorylated state of that receptor. To test the hypothesis that the low affinity class of progesterone binding found in the particulate fraction of T47D cells might represent a phosphorylated form of receptor which is converted to high affinity form by endogenous phosphatase activity during solubilization, the particulate fraction was extracted in buffers containing 1 μM glucose 1-phosphate, a saturating concentration for inhibition of phosphatase activity. The dissociation constant and receptor number for [^{3}H]progesterone binding to either the particulate or soluble (cytosol) fraction were not altered by including glucose 1-phosphate (data not shown). Moreover, buffers used for cell homogenization and high ionic strength extraction of the particulate fraction contained 10 mM sodium molybdate, a known phosphatase inhibitor (23).

Determination of Dissociation Constants for High and Low Affinity-specific Progesterone Binding from Rate Measurements—Rates of progesterone association to and dissociation from T47D cells were studied at 0 °C to determine if the two classes of progesterone binding observed at equilibrium could also be distinguished by their kinetic properties. The kinetics of progesterone binding to high affinity sites were studied at 1 nM [^{3}H]progesterone, the equilibrium dissociation constant for high affinity binding. The kinetics for binding to low affinity sites were studied at 50 nM [^{3}H]progesterone, their approximate equilibrium K_d. At 1 nM [^{3}H]progesterone, both association and dissociation kinetics for progesterone binding revealed a single class of receptors (Fig. 3, *A* and *B*). The dissociation constant (k_d) determined from the ratio of the dissociation rate constant ($k_d = 1.13 \times 10^{-3}$ min^{-1}) to the association rate constant ($k_a = 23.3 \times 10^{-3}$ nM^{-1}·min^{-1}) was 0.05 nM (Table V); this value is 20-fold lower than the equilibrium K_d for the high affinity sites (Table I). Studies on rates of association at 50 nM [^{3}H]progesterone, a concentration where both sites would be occupied, revealed two components, fast and slow (Fig. 3*C*). Based on the progesterone binding kinetics at 1 nM [^{3}H]progesterone, where predominantly high affinity sites are occupied, the fast and slow associating components seen at 50 nM [^{3}H]progesterone are bindings to high and low affinity sites, respectively. Similarly, dissociation of progesterone from T47D cells incubated with 50 nM [^{3}H]progesterone was biphasic with quickly and more slowly dissociating components (Fig. 3*D*). The ratio of the rate constant of the slowly dissociating component (0.51×10^{-3} min^{-1}) to the rate constant of the rapidly associating

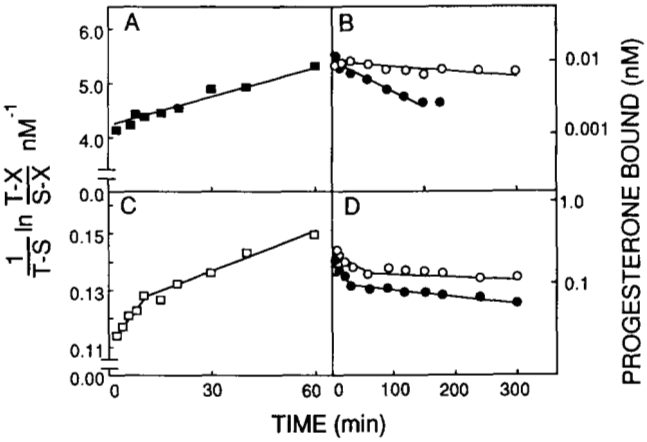

FIG. 3. **Rates of progesterone association with or dissociation from T47D cells.** Association rates of progesterone binding to T47D cells were determined by incubating cells at 0 °C in 0.5 ml of binding medium containing 1 or 50 nM [^{3}H]progesterone for the times indicated. Receptor-associated [^{3}H]progesterone was determined as described for Fig. 1 by subtracting nonspecific binding occurring in the presence of 10 μM unlabeled progesterone. Dissociation of [^{3}H]progesterone from adherent T47D cells was determined by first incubating the cells in 0.5 ml of binding medium with either 1 or 50 nM [^{3}H]progesterone for 60 min at 22 °C and then at 0 °C for 30 min. Following three rapid washes at 0 °C, dissociation of [^{3}H]progesterone was initiated by adding 0.5 ml of binding medium and incubating at 0 °C. After binding with [^{3}H]progesterone, one set of samples was incubated in binding medium containing 5 μM cold progesterone to determine dissociation. At the time points indicated, binding medium containing dissociated [^{3}H]progesterone was removed, and the cells were processed for specific bound [^{3}H]progesterone as described for Fig. 1. The association rate was determined from the slope of the integrated form of the second-order reaction, where X = concentration of receptor-[^{3}H]progesterone complex at each time measured, T = initial concentration of [^{3}H]progesterone, and S = initial concentration of receptor-binding sites, *i.e.* concentration of receptor-bound [^{3}H]progesterone at saturation as described by Schrader and O'Malley (8). Dissociation rates were determined from the slopes of dissociation plots. *A* and *B*, [^{3}H]progesterone association with or dissociation from cells incubated with 1 nM [^{3}H]progesterone, respectively; *C* and *D*, association and dissociation at 50 nM [^{3}H]progesterone, respectively. In *B* and *D*, the *filled circles* represent experiments where dissociation of progesterone occurred in the presence of 5 μM unlabeled progesterone. The scale of the *ordinate* for *B* and *D* is logarithmic. The data express binding/10^5 cells.

TABLE V

Rate constants for progesterone binding to adherent T47D cells

Data were obtained from the experiment described for Fig. 3. Numbers in parentheses are values obtained in the presence of 5 μM unlabeled progesterone.

Progesterone	High affinity			Low affinity		
	k_a	k_d	K_d (k_d/k_a)	k_a	k_d	K_d (k_d/k_a)
nM	$\times 10^{-3}$ nM$^{-1}\cdot$min^{-1}	$\times 10^{-3}$ min^{-1}	nM	$\times 10^{-3}$ nM$^{-1}\cdot$min^{-1}	$\times 10^{-3}$ min^{-1}	nM
50.0	1.27	0.51	0.40	0.48	30	63
		(1.7)	(1.3)		(52)	(108)
1.0	23.3	1.13	0.05	ND[a]	ND	ND
		(9.71)	(0.42)			

[a] ND, not detectable.

component (1.27×10^{-3} nM$^{-1}\cdot$min^{-1}), *i.e.* those rate constants representing dissociation from and association to high affinity sites, respectively, yielded a rate-determined dissociation constant of 0.4 nM; and this approaches the K_d value of 1.5 nM obtained for high affinity progesterone equilibrium binding (Table V). The ratio of the rate constant of the rapidly dissociating component (30×10^{-3} min^{-1}) to the rate constant of the slowly associating component (0.48×10^{-3} nM$^{-1}\cdot$min^{-1}) yielded a rate-determined dissociation constant of 63 nM, similar to the equilibrium K_d for low affinity progesterone-binding sites (Table V).

The rate-determined dissociation constant for high affinity sites (Table V; K_d = 0.05 nM) was 20-fold lower than the equilibrium K_d for these sites (Table I). This discrepancy may be due to reassociation of [^{3}H]progesterone dissociated from high affinity sites. Therefore, the dissociation rate of [^{3}H]progesterone bound at 1 nM to high affinity sites was studied in the presence of 5 μM unlabeled progesterone to prevent reassociation of dissociated [^{3}H]progesterone (Fig. 3*B* and Table V (values in parentheses)). In this case, the rate of dissociation of [^{3}H]progesterone was faster than that observed in the absence of unlabeled progesterone, and the dissociation constant obtained by calculation from the rate constants was 0.42 nM (Table V), in closer agreement with the equilibrium K_d (Table I) for high affinity binding. Similarly, the dissociation rate of [^{3}H]progesterone bound specifically to cells incubated with 50 nM [^{3}H]progesterone was 2–3-fold faster in the presence of 5 μM unlabeled progesterone than in the absence of unlabeled progesterone (Fig. 3*D* and Table V). Under this condition, the rate-determined dissociation constants for high and low affinity binding sites were in close agreement with their respective equilibrium dissociation constants. Thus, kinetic studies revealed two classes of progesterone binding, high and low affinity, similar in properties to those obtained in equilibrium binding studies (compare Tables I and V).

Extraction of Progesterone Receptor-Progesterone Complexes from Cells with Nonionic Detergent—Since Triton X-100 treatment of adherent cells leaves the nuclei, the cytoskeletal residue, and the extracellular matrix attached to the culture dish (20), it provided an opportunity to study rates of release of high affinity hormone receptor complexes from cells. T47D cells were incubated with 1, 5, or 50 nM [^{3}H]progesterone until equilibrium binding was achieved. Then, cells were washed and treated with nonionic detergent at 0 °C for varying time periods. At all three concentrations of progesterone, one component was rapidly extracted with a half-time of approximately 1 min (Fig. 4, *inset*). Extraction of this component was essentially complete by 5 min. In addition, a more slowly extracting component with a half-time of about 25 min at 0 °C was observed for cells incubated with 5 or 50 nM [^{3}H]progesterone. This half-time is approximately the same as the half-time of 20 min observed for dissociation of [^{3}H]progesterone from the low affinity sites in the absence of detergent (Fig. 3*D*). Therefore, the component dissociating slowly upon nonionic detergent extraction most likely represents dissociation of progesterone from the low affinity sites in the cellular residue remaining adherent to the culture dish (20). At 1 nM [^{3}H]progesterone, where high affinity sites are occupied predominantly, there was no appreciable amount (<3% of cellular specific bound progesterone) of this more slowly extracting component.

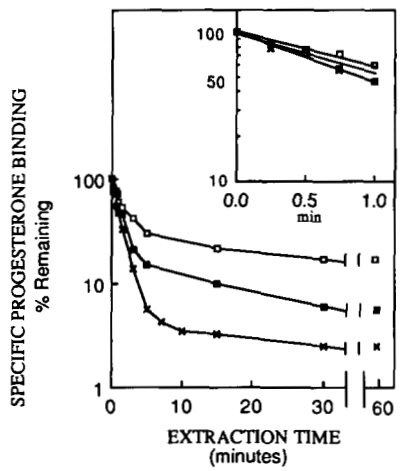

FIG. 4. **Rate of nonionic detergent extraction of [^{3}H]progesterone specifically bound to high or low affinity sites.** Adherent T47D cells were incubated in binding medium with 1, 5, or 50 nM [^{3}H]progesterone for 60 min at 23 °C. Replicate samples were incubated with 10 μM unlabeled progesterone to determine nonspecific binding. The cells were washed as described for Fig. 1 and treated with nonionic detergent (binding medium containing 0.5% (w/v) Triton X-100) at 4 °C with continuous gentle shaking for the time periods indicated. Specifically bound [^{3}H]progesterone in the detergent extract or residue was then quantified (see "Experimental Procedures"). Percent specific bound progesterone remaining was calculated by subtracting specifically bound [^{3}H]progesterone in the detergent extract from total cellular specifically bound [^{3}H]progesterone. ×, ■, and □, percent specific progesterone binding remaining in cells incubated with 1, 5, and 50 nM [^{3}H]progesterone, respectively.

β-Acetylglucosaminidase, a lysosomal enzyme, was extracted rapidly from T47D cells by Triton X-100 with a half-time of 1 min, with a negligible slowly extracting component (<2% of cellular β-acetylglucosaminidase activity). These extraction kinetics of β-acetylglucosaminidase were identical to those of high affinity progesterone binding (Fig. 4, binding at 1 nM [^{3}H]progesterone). In contrast, low affinity progesterone receptors (Fig. 4, progesterone binding at 50 nM) were resistant to nonionic detergent extraction under conditions where a membrane-compartmentalized protein, such as lysosomal β-acetylglucosaminidase, was extracted readily.

Time courses for progesterone binding detected subsequently in the nonionic detergent-extractable and residue fractions were studied to determine whether rates of progesterone binding to these fractions differed as predicted by the kinetics of progesterone association to or dissociation from high and low affinity sites. Adherent T47D cells were incubated at 22 or 37 °C with either 1 or 100 nM [^{3}H]progesterone, washed with ice-cold buffer, and extracted with nonionic detergent solution at 0 °C for 5 min. Since 5 min of nonionic detergent treatment completely extracted the high affinity class of binding (Fig. 4), cells were extracted with Triton X-100 for 5 min, and specifically bound progesterone in the detergent extract and residue was determined.

Progesterone binding reached saturation rapidly in the nonionic detergent-extractable fraction, whereas appearance of hormone in the detergent residue fraction was slower (Fig. 5, *A–D*), consistent with the presence of high and low affinity progesterone-binding components. The slower appearance of progesterone in the nonionic detergent residue fraction was particularly evident when the incubations were done at 100 nM progesterone and at 22 °C rather than at 37 °C (Fig. 5, compare *A* and *B*). At 1 nM progesterone, binding was observed predominantly in the nonionic detergent-extractable fraction, with very little binding in the residue fraction (Fig. 5, *C* and *D*). These data demonstrate that the nonionic detergent-extractable fraction contains rapidly saturable progesterone-binding sites and suggest that these are the high affinity receptors, whereas the detergent residue fraction contains slowly saturable progesterone-binding sites, a property expected of low affinity receptors.

Resolution of High Affinity from Low Affinity Progesterone Receptors by Extraction with Nonionic Detergent—The data presented above suggest that nonionic detergent resolves high and low affinity progesterone receptors rapidly. To test this hypothesis rigorously, adherent T47D cells were incubated with [^{3}H]progesterone over a wide range of concentrations.

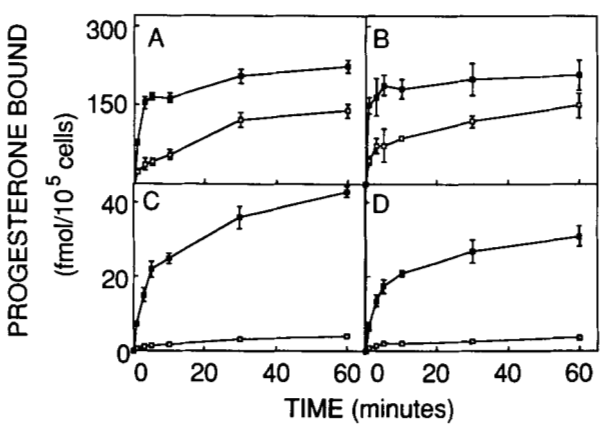

FIG. 5. **Time course for saturation of nonionic detergent-extractable and -nonextractable cellular progesterone receptors.** Adherent T47D cells were incubated *in situ* in 0.5 ml of binding medium with 100 nM [^{3}H]progesterone at 22 °C (*A*) or 37 °C (*B*) or with 1 nM [^{3}H]progesterone at 22 °C or 37 °C (*D*) for the times indicated. The cells were then washed with binding medium at 4 °C to remove free progesterone and treated with binding medium containing 0.5% nonionic detergent (Triton X-100) for 5 min with gentle shaking (see "Experimental Procedures"). The supernatant solution was removed, and the detergent-nonextractable residue was solubilized in 1 M NaOH. Aliquots were processed for determination of radioactivity (see "Experimental Procedures"). The figures show specifically bound [^{3}H]progesterone ± S.D. (n = 4) in the supernatant (■) and residue (□) fractions/10^5 cells. Nonspecific binding occurring in the presence of 10 μM unlabeled progesterone is deducted from all values.

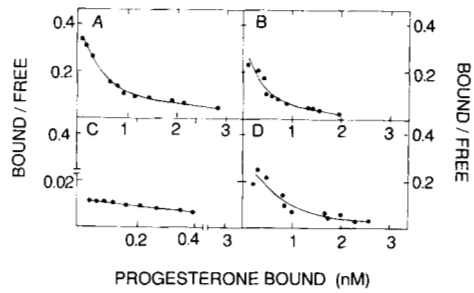

FIG. 6. **Analysis of specifically bound [^{3}H]progesterone in nonionic detergent-extractable and -nonextractable fractions with T47D cells incubated with [^{3}H]progesterone.** Adherent T47D cells were incubated with 0.25–150 nM [^{3}H]progesterone at 23 °C for 60 min and processed as described for Fig. 1, except that specifically bound [^{3}H]progesterone to cells was determined either directly by NaOH treatment or by nonionic detergent extraction for 5 min at 0 °C followed by NaOH solubilization of the detergent residue (see "Experimental Procedures"). Data are specifically bound [^{3}H]progesterone in cells solubilized directly by NaOH (*A*), in the nonionic detergent extract (*B*), or in the nonionic detergent-nonextractable fractions (*C*). The combined data from *B* + *C* are shown in *D*. Scatchard plots were computed from raw data using the LIGAND program of Munson and Rodbard (22). Each point is the mean of three independent experiments.

After equilibrium binding was achieved, cells were extracted with 0.5% Triton X-100 for 5 min at 0 °C to yield a detergent-extractable supernatant fraction and a detergent-nonextractable residue. This period of incubation with nonionic detergent achieved complete solubilization of the rapidly extracting component, presumably the high affinity complex of progesterone with its receptor (Fig. 4). Specifically bound progesterone in both fractions was characterized by the LIGAND program (22). In cells not extracted with detergent, both high and low affinity progesterone-binding sites were revealed (Fig. 6*A*). Similarly, the detergent-extractable material also contained radioactive progesterone derived from sites at both affinities (Fig. 6*B*). The presence of some of the low affinity component in the detergent extract was expected. By 5 min of detergent extraction of cells, significant dissociation of progesterone from the slowly extracting component was also observed (Fig. 4).

The detergent-nonextractable residue contained low affinity sites exclusively (Fig. 6*C*). A Scatchard plot of the combined radioactivities in the Triton-extractable plus -nonextractable fractions was essentially identical to that obtained by analysis of cells not extracted with detergent (Fig. 6*D*). The values of dissociation constants for progesterone bound at high or low affinity obtained following detergent extraction were similar to those obtained with cells not treated with detergent (Table VI). These data indicate that the high affinity class of progesterone receptors is readily extracted by nonionic detergent, whereas the low affinity class of receptors is not.

DISCUSSION

Two classes of equilibrium progesterone binding, high and low affinity, have been demonstrated in substratum-adherent cultured human breast carcinoma T47D cells (Fig. 1 and Table I). The low affinity class of receptors comprise most of the cellular progesterone binding. The demonstration of a low affinity class of progesterone receptors in intact T47D cells, exclusive localization of these receptors in the particulate fraction, and their conversion from a low affinity particulate form to a high affinity form upon solubilization are novel findings.

Progesterone metabolism at 37 °C by intact T47D cells has

TABLE VI

Partitioning of specific equilibrium bound [^{3}H]progesterone in T47D cells upon extraction with nonionic detergent

Values for K_d and receptor number were computed from the data obtained in the experiment described for Fig. 6 and from two independent experiments using the LIGAND program of Munson and Rodbard (22). Values are means from these three experiments ± S.D.

Fraction	Dissociation constant		Receptor number	
	K_{d1}	K_{d2}	R_1	R_2
	nM		*×10^6 sites/cell*	
Whole cell	60 ± 8	1.5 ± 0.5	2.4 ± 1.0	0.33 ± 0.15
Detergent-extractable	60 ± 13	1.6 ± 0.2	1.2 ± 0.4	0.25 ± 0.15
Detergent-nonextractable	88 ± 20	ND[a]	0.8 ± 0.1	ND[a]
Combined (B + C)	69 ± 22	2.2 ± 1.6	1.7 ± 0.8	0.35 ± 0.21

[a] ND, none detected.

been reported to occur with a half-time of 2 h (24). Under the conditions we used to study equilibrium binding (22 °C for 60 min), intact T47D cells metabolized less than 20% of the [^{3}H] progesterone present in the medium at either high or low progesterone concentration (see Miniprint). Moreover, incubation of soluble or particulate fractions of cell homogenates at 4 °C with high or low concentrations of [^{3}H]progesterone revealed no significant metabolism in the [^{3}H]progesterone bound specifically to either soluble (high affinity) or particulate (low affinity) receptors (see Miniprint).

In analyzing progesterone binding to adherent cells or cell homogenates, we employed a computer program (22) that uses a statistically valid curve-fitting algorithm with objective measurement of goodness of fit to discriminate between a single class and two classes of ligand binding. Nonspecific [^{3}H]progesterone binding was determined by two independent methods: (i) experimentally, from replicate samples incubated with [^{3}H]progesterone and 10 μM unlabeled progesterone, and this gave linear nonspecific binding (Fig. 1*A*); and (ii) computationally, using the LIGAND program created by Munson and Rodbard (22).

The evidence for two classes of progesterone binding from equilibrium data is supported by rate studies which establish two classes of receptor based on calculations from rate constants determined for ligand association or dissociation (Fig. 1 and Table I; Fig. 2 and Table IV; Fig. 3 and Table V). The values for dissociation constants derived from equilibrium or rate approaches are in substantial agreement and indicate a high affinity class of progesterone binding of K_d = 1.5–2.0 nM and a low affinity class of K_d = 20–60 nM. Whereas we find that dissociation constants for progesterone binding to adherent T47D cells obtained from rate and equilibrium binding studies are in agreement, other investigators (8, 25) have reported that the K_d derived from rate studies for progesterone binding to chick oviduct cytosol or human myometrium cytosols is 10–50-fold lower than the equilibrium binding K_d.

Two classes of progesterone binding have been observed in the cytosol from human breast carcinoma MCF-7 cells (13). We find that in T47D cells, progesterone binding in the soluble (cytosol) fraction consists entirely of the high affinity class, whereas the low affinity class is exclusively in the particulate fraction of a cell homogenate (Fig. 2 and Table IV). Based on MCF-7 cell data showing that progestins competed for [^{3}H]dexamethasone binding, but that dexamethasone failed to compete for [^{3}H]progesterone binding, Lippman *et al.* (13) concluded that the low affinity class of progesterone binding in MCF-7 cells was on the glucocorticoid receptor, possibly on an allosteric site. Rousseau *et al.* (26) and Jones and Bell (27) also demonstrated low affinity progesterone binding to glucocorticoid receptor, and dexamethasone did not compete with that binding. Because MCF-7 cells have twice as many glucocorticoid receptors as they do progesterone receptors (16), we used T47D cells, which have marginal levels of glucocorticoid receptors as determined by [^{3}H]dexamethasone binding, approximately 10^4 sites/cell (determined by us, not shown, and Ref. 16), as compared with the extraordinarily high levels of progesterone receptors present (~2 × 10^6 sites/cell; Table I). Moreover, the low affinity progesterone-binding sites in T47D cells sedimented with the particulate fraction of cell homogenates (Table IV), whereas glucocorticoid receptors are in the soluble (cytosolic) fraction (26, 27). The K_d for progesterone binding to T47D cell receptor solubilized from the particulate fraction at high ionic strength was approximately 2 nM; this value is not in agreement with progesterone binding to glucocorticoid receptor at a K_d of 30 nM (26). Competition binding with progestins and other steroids showed that the high and low affinity classes of progesterone-binding sites in T47D cells were highly specific for progestins (Tables VIII and IX) and therefore represent progesterone receptors. High and low affinity hormone-binding sites have also been reported for estradiol in rat uterus (28) and for insect juvenile hormone in fat bodies of the cockroach (29). However, in both those cases, the two classes of binding sites were found in the soluble fraction of a cell homogenate.

High affinity progesterone receptors are found exclusively in the soluble fraction prepared either by nonionic detergent extraction or by homogenization of cells, whereas the low affinity receptors are in the particulate residue with either method of extraction. Light microscopic examination of the particulate fraction obtained by either detergent extraction or homogenization of cells revealed nuclei surrounded by fibrous material. Our procedure for nonionic detergent extraction of cells is based on the method of Brown *et al.* (20), who have shown, by both light and electron microscopy, that with nonionic detergent-extracted adherent cells, nuclei, the cytoskeleton, and the extracellular matrix remain attached to the cell culture dish. Thus, the low affinity particulate receptors may be associated with one or more of these structures. Our biochemical studies are in agreement with a finding by Perrot-Applanat *et al.* (7), who showed by immunocytochemistry at the electron microscopic level that progesterone receptors in rabbit uterine stromal cells are localized predominantly in the nucleus with fewer receptors in the cytoplasm, where they are associated with rough endoplasmic reticulum and free ribosomes. More recently, using immunocytochemistry, this observation was confirmed in T47D cells by El-Ashry and Edwards (30) and in uterine epithelial cells, uterine stromal smooth muscle cells, and breast ductal cells by Press and Greene (31).

We have observed that exposure of our T47D cell strain to progesterone did not lead to any detectable redistribution of progesterone receptors from a soluble to particulate form. The reasons why our data on progesterone receptor translocation are in conflict with those of Mockus and Horwitz (4) are not clear since we duplicated their method exactly. The differences between our data and theirs could reside in the different strains of T47D cells used by us and by Mockus and Horwitz (4). However, it is important to note that El-Ashry and Edwards (30) failed to detect progesterone receptor redistribution by immunocytochemical staining following exposure of T47D cells to progesterone. Moreover, Ennis *et al.* (32) were unable to demonstrate, by immunocytochemistry, cytoplasmic to nuclear translocation of progesterone receptors following exposure of chick oviduct *in vitro* to progesterone.

Our data likewise indicate that most of the progesterone receptors in T47D cells are nuclear, that they exist in a low affinity form, and that physiologic concentrations of progesterone cause no detectable redistribution.

Both high and low affinity progesterone receptors appear to be either the same or a closely related protein. Solubilization of the low affinity, particulate fraction-associated receptors in buffers at high ionic strength rendered their binding properties similar to those of high affinity receptors fractionating with the cytosol (Fig. 2, and Table IV). Steroid competition binding studies indicated that both affinity classes of receptors had similar steroid binding specificities (Table IX). If they are the same or a related protein, then the low affinity property must be related to the association of these receptors with a subcellular particulate structure. Low affinity progesterone receptors may not have been observed in earlier investigations because intact cells were not used for determination of dissociation constants (13, 15). Instead, the cytosol exclusively was used, and this contains only the high affinity form.

High and low affinity progesterone-binding sites have been observed with purified chick oviduct progesterone receptor (12). The two classes of progesterone-binding sites in T47D cells differ from those of chick oviduct progesterone receptor in that in the latter case, both classes of binding are associated with soluble proteins; whereas in the former case, high and low affinity progesterone binding derives from receptor being present in the soluble or particulate compartments, respectively. Experiments by Maggi *et al.* (12) suggest that in chick oviduct progesterone receptor, low affinity binding results from receptor phosphorylation. Alkaline phosphatase destroyed the low affinity binding, and glucose 1-phosphate protected this site from phosphatase action. In T47D cells, solubilization of low affinity particulate progesterone receptors by high ionic strength buffer converted them to a high affinity form; this change in affinity was not prevented by including phosphatase inhibitors during extraction.

The demonstration of distinct high and low affinity classes of progesterone binding raises the issue of possibly unique functional roles. Ultrastructural studies reveal the nucleus-associated form of both progesterone and estrogen receptors as the major component (5–7), and our findings support that conclusion. Since the low affinity progesterone receptors are both more abundant and more avidly associated with the particulate fraction (Figs. 1 and 2 and Table I–VI), they are likely to represent that nucleus-associated form revealed by immunocytochemical methodology. The nature of the association is not known. Treatment with high ionic strength solutions both extracts the low affinity form from the particulate fraction and converts it to an affinity characteristic of the soluble receptor. It will be of interest to determine if the low affinity form arises from a cellular modification of high affinity receptors and also if specific macromolecules in the particulate fraction are responsible for the creation and/or maintenance of the low affinity state.

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REFERENCES

1. Jänne, O., Kontula, K., Vihko, R., Feil, P. D., and Bardin, C. W. (1978) *Med. Biol. (Helsinki)* **56,** 225–248
2. Grody, W. W., Schrader, W. T., and O'Malley, B. W. (1982) *Endocr. Rev.* **3,** 141–163
3. Walters, M. R. (1985) *Endocr. Rev.* **6,** 512–543
4. Mockus, M. B., and Horwitz, K. B. (1983) *J. Biol. Chem.* **258,** 4778–4783
5. King, W. J., and Green, G. L. (1984) *Nature* **307,** 745–747
6. Welshons, W. V., Lieberman, M. E., and Gorski, J. (1984) *Nature* **307,** 747–749
7. Perrot-Applanat, M., Groyer-Picard, M. T., Logeat, F., and Milgrom, E. (1986) *J. Cell Biol.* **102,** 1191–1199
8. Schrader, W. T., and O'Malley, B. W. (1972) *J. Biol. Chem.* **247,** 51–59
9. Schrader, W. T., Kuhn, R. W., and O'Malley, B. W. (1977) *J. Biol. Chem.* **252,** 299–307
10. Coty, W. A., Schrader, W. T., and O'Malley, B. W. (1979) *J. Steroid Biochem.* **10,** 1–12
11. Birnbaumer, M. E., Schrader, W. T., and O'Malley, B. W. (1983) *J. Biol. Chem.* **258,** 1637–1644
12. Maggi, A., Schrader, W. T., and O'Malley, B. W. (1984) *J. Biol. Chem.* **259,** 10956–10966
13. Lippman, M., Huff, K., and Bolan, G. (1977) *Ann. N. Y. Acad. Sci.* **286,** 101–115
14. Horwitz, K. B., Costlow, M. E., and McGuire, W. L. (1975) *Steroids* **26,** 785–795
15. Mockus, M. B., Lessey, B. A., Bower, M. A., and Horwitz, K. B. (1982) *Endocrinology* **110,** 1564–1571
16. Horwitz, K. B., Zava, D. T., Thilagar, A. K., Jensen, E. M., and McGuire, W. L. (1978) *Cancer Res.* **38,** 2434–2437
17. Ghosh-Dastidar, P., Coty, W. A., Griest, R. E., Woo, D. D. L., and Fox, C. F. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 1654–1658
18. Woo, D. D. L., Fay, S. P., Griest, R., Coty, W., Goldfine, I., and Fox, C. F. (1986) *J. Biol. Chem.* **261,** 460–467
19. Seematter, R. J., Hoffman, P. G., Kuhn, R. W., Lockwood, L. C., and Siiteri, P. K. (1978) *Cancer Res.* **38,** 2800–2805
20. Brown, S., Levinson, W., and Spudich, J. A. (1976) *J. Supramol. Struct.* **5,** 119–130
21. Baggiolini, M. (1974) *Methods Enzymol.* **31,** 345–353
22. Munson, P. J., and Rodbard, D. (1980) *Anal. Biochem.* **107,** 220–239
23. Hollander, V. P. (1971) in *The Enzymes* (Boyer, P. D., ed) Vol. 4, pp. 449–498, Academic Press, New York
24. Horwitz, K. B., Mockus, M. B., Pike, A. W., Fennessey, P. V., and Sheridan, R. L. (1983) *J. Biol. Chem.* **258,** 7603–7610
25. Jänne, O., Kontula, K., and Vihko, R. (1976) *J. Steroid Biochem.* **7,** 1061–1068
26. Rousseau, G. G., Baxter, J. D., and Tomkins, G. M. (1972) *J. Mol. Biol.* **67,** 99–115
27. Jones, T. R., and Bell, P. A. (1980) *Biochem. J.* **188,** 237–245
28. Clark, J. H., Hardin, J. W., Upchurch, S., and Eriksson, H. (1978) *J. Biol. Chem.* **253,** 7630–7634
29. Engelmann, F. (1981) *Mol. Cell. Endocr.* **24,** 103–112
30. El-Ashry, D., and Edwards, D. P. (1987) *69th Annual Meeting of the Endocrine Society,* Indianapolis, IN, Abstr. 181
31. Press, M. F., and Greene, G. L. (1987) *69th Annual Meeting of the Endocrine Society,* Indianapolis, IN, Abstr. 179
32. Ennis, B. W., Stumpf, W. E., Gasc, J. M., and Baulieu, E. E. (1986) *Endocrinology* **119,** 2066–2075
33. Strott, C. A. (1974) *Endocrinology* **95,** 826–837

Resolution of High and Low Affinity Progesterone Receptors from Human Breast Carcinoma T47D Cells

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Progesterone metabolism

Since [^{3}H]progesterone metabolites could influence progesterone binding if formed *de novo* in intact T47D cells or in cell homogenates, tests for progesterone metabolism were performed under conditions used for the binding experiments. Intact T47D cells and the soluble or particulate fractions of homogenates from T47D cells were incubated with [^{3}H]progesterone at 1 nM (Kd for high affinity binding) or at 100 nM (twice the Kd for low affinity binding) under conditions (Figs. 1, 2) where two classes of progesterone receptors were observed. [^{3}H]Ligand bound to the soluble or particulate fraction and that remaining in the incubation medium were characterized by thin layer chromatography following the procedure of Strott (33).

Between 93 to 96 % of radioactivity in [^{3}H]progesterone not exposed to cells had the mobility of progesterone in two independent solvent systems [benzene : ethyl acetate (8 : 2) or ethyl acetate : chloroform (50 : 1)] when analyzed by thin layer chromatography (Fig. 7 A, Fractions 3 to 6, Table VII). Of the 7 % impurities in the input radioactivity, 2 % remained at the origin (Fig. 7 A, Fraction 1), 2 % was in fraction 2 and the remaining 3 % was distributed over fractions 7 to 10 (Fig. 7 A).

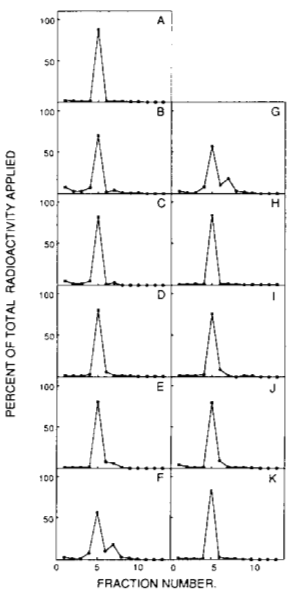

Fig. 7. Thin layer chromatographic analysis of [^{3}H]progesterone bound to T47D cells.

Adherent T47D cells, grown on 55 cm^2 petri plates, were incubated at 22° for 60 min with 1 or 100 nM [^{3}H]progesterone as described in the Fig. 1 legend. The medium was reserved, and the cells were then washed and suspended in homogenization buffer at 0°. The soluble and particulate fractions were prepared (*Experimental Procedures*). Both these fractions and the incubation medium were extracted three times with ethyl acetate. This procedure extracted > 99 % of radioactivity present in the soluble or particulate fraction and no detectable radioactivity remained in the ethyl acetate extracted particulate residue. The ethyl acetate extracts were analyzed for [^{3}H]progesterone and its metabolites by silica gel thin layer chromatography in benzene : ethyl acetate (8 : 2) following the procedure of Strott (33). In parallel experiments, soluble and particulate fractions resolved from a T47D cell homogenate were incubated at 4° for 16 h as described in the legend of Fig. 2 with 1 or 100 nM [^{3}H]progesterone, and ethyl acetate extracts of ligand bound to these fractions were analyzed for [^{3}H]progesterone and its metabolites as described above. Following chromatography, sample lanes were scraped in 1.0 cm fractions which were quantified for radioactivity. Fractions 1 and 13 represent origin and solvent front, respectively.

(A) [^{3}H]progesterone used for the incubations;
(B) and (C) medium from cells incubated with 1 or 100 nM [^{3}H]progesterone, respectively;
(D) and (E) soluble and particulate fractions, respectively, from cells incubated with 1 nM [^{3}H]progesterone;
(F) and (G) soluble and particulate fractions, respectively, from cells incubated with 100 nM [^{3}H]progesterone;
(H) and (I) resolved soluble and particulate fractions, respectively, incubated with 1 nM [^{3}H]progesterone;
(J) and (K) resolved soluble and particulate fractions, respectively, incubated with 100 nM [^{3}H]progesterone.

TABLE VII. Progesterone metabolism by intact T47D cells and by their cell homogenates.*

	Incubation Condition	[^{3}H]Progesterone Concentration (nM)	Unmetabolized [^{3}H]progesterone (% Total Radioactivity) Fraction Chromatographed	Benzene-Ethyl acetate Solvent	Ethyl acetate:Chloroform Solvent
A	No incubation	100	Input	93	96
			[^{3}H]Progesterone		
B	Adherent Cells	1	Medium	82	91
C		100	Medium	90	95
D		1	Soluble binding	90	91
E		1	Particulate "	91	94
F		100	Soluble "	82	95
G		100	Particulate "	76	94
H	Cell Homogenate	1	Soluble binding	90	95
I		1	Particulate "	90	97
J		100	Soluble "	90	96
K		100	Particulate "	93	95

* These data were obtained from the experiment described in Fig. 7 and from replicate samples chromatographed in ethyl acetate : chloroform (50 : 1) solvent as described in the Fig. 7 legend. Values are % of total radioactivity applied for thin layer chromatography.

Tests for [^{3}H]progesterone metabolism by cells

Analysis of medium from cells incubated with 1 or 100 nM [^{3}H]progesterone revealed that > 80 % of the labelled progesterone was not metabolized (Table VII). There were two major progesterone metabolites in the medium : one (approximately 10 % of input radioactivity) remained at the origin (Fig. 7 B, C, Fraction 1) and the other (approximately 5 % of input radioactivity) was recovered in fractions 7 and 8. Analysis of radioactivity bound to receptors in the soluble or particulate fractions prepared from cells incubated with 1 nM [^{3}H]progesterone revealed that > 90 % of the radioactivity bound to either fraction was unmetabolized [^{3}H]progesterone (Table VII). The major metabolite(s) present in either the soluble or particulate fraction was in Fractions 7 and 8 (Fig. 7 D, E) and represented approximately 8 % of input radioactivity. Over 80 % of the radioactivity bound to these same fractions derived from cells incubated with 100 nM [^{3}H]progesterone was unmetabolized [^{3}H]progesterone (Fig. 7 G, H, Table VII), and the major metabolite(s) amounting to 13 - 20 % of input radioactivity was found in fractions 7 and 8 (Fig. 7 G, H).

Tests for [^{3}H]progesterone metabolism by isolated subcellular fractions

When resolved soluble or particulate fractions were incubated with 1 or 100 nM [^{3}H]progesterone under conditions described for Fig. 2, no evidence for [^{3}H]progesterone metabolism was detected (Fig 7 H - K, Table VII).

Steroid binding specificity of high and low affinity progesterone receptors

Competition for [^{3}H]progesterone binding by unlabelled progestins and other steroids was studied to determine if both low and high affinity progesterone binding sites had the same or different specificities for ligand binding. Competition for high affinity progesterone binding by other steroids was studied by incubating adherent T47D cells with 5 nM [^{3}H]progesterone with the unlabelled steroids absent or present, and competition for the low affinity sites was examined at 50 nM [^{3}H]progesterone. Only progestins, namely progesterone, R5020, and MPA, in that order of potency, competed effectively for both high and low affinity [^{3}H]progesterone binding (Table VIII). The androgen 5 -α-dihydrotestosterone, which is reported to have activity for translocating progesterone receptors and to have progestin effects (1) was weakly competitive with progesterone for its binding to either high or low affinity progesterone receptors.

TABLE VIII Competition of [^{3}H]progesterone binding to intact T47D cells by steroids

T47D cells were incubated at 22° for 60 min in binding medium containing 5 nM [^{3}H]progesterone or 50 nM [^{3}H]progesterone and unlabelled steroids as indicated. Control incubations contained [^{3}H]progesterone alone. Nonspecific binding was determined from replicate samples incubated with [^{3}H]progesterone and 5 μM unlabelled progesterone. At the end of the incubations, cells were washed and processed for determination of specifically bound [^{3}H]progesterone (*Experimental Procedures*). Values are means of five independent determinations.

Additions	(nM)	[^{3}H]Progesterone Specific Binding * 5 nM [^{3}H]Progesterone	50 nM [^{3}H]Progesterone
None		100	100
Progesterone	100	32	67
	5,000	6	8
R5020	100	28	55
	5,000	8	16
Medroxyprogesterone Acetate	100	43	69
	5,000	36	59
Dexamethasone	100	100	100
	5,000	62	73
17-ß-Estradiol	100	94	100
	5,000	50	68
5-α-Dihydrotestosterone	100	83	92
	5,000	28	49
Aldosterone	100	100	100
	5,000	50	66

* Specific [^{3}H]progesterone binding to T47D cells in the absence of added steroid is expressed as 100 % and were 551,000 ± 50,000 sites/cell at 5 nM [^{3}H]progesterone and 1,650,000 ± 70,000 sites/cell at 50 nM [^{3}H]progesterone.

Steroid binding specificity also was studied with soluble and particulate progesterone receptors, the latter fraction following their solubilization at high ionic strength. Receptors extracted from the particulate fraction by high ionic strength buffers acquire the same high affinity as receptors which fractionated with the cytosol. Both were incubated with 5 nM [^{3}H]progesterone to study competition at 10- or 100-fold excess of unlabelled steroids. Both the cytosol- and particulate- fraction derived progesterone receptors had a high degree of specificity for the progestins, progesterone, R5020, and MPA (Table IX). Other steroids were only weakly competitive at 100-fold excess. These data demonstrate that both high and low affinity progesterone binding sites in T47D cells are specific for progestins and that they are similar in their steroid binding specificities either prior to or after extraction of the low affinity form from the particulate fraction.

TABLE IX. Competition of [^{3}H]progesterone binding to soluble and particulate progesterone receptors by steroids.

The 40,000 g.min soluble and particulate fractions of T47D cell homogenates were prepared and the particulate fraction was extracted with 0.5 M KCl as described in *Experimental Procedures*. The soluble fraction and the high ionic strength extract of the particulate fraction were adjusted to 0.25 M KCl in homogenization buffer and incubated with 5 nM [^{3}H]progesterone and unlabelled steroids as indicated. For determination of nonspecific binding, replicate samples also contained 5 μM unlabelled progesterone. Receptor bound [^{3}H]progesterone was determined by the dextran-coated charcoal assay (*Experimental Procedures*). Values are means of three independent determinations.

Additions	nM	[^{3}H]Progesterone Specific Binding * Soluble Fraction	Extract of Particulate Fraction
None		100	100
Progesterone	50	14	15
	500	4	1
R5020	50	13	9
	500	4	5
Medroxyprogesterone Acetate	50	23	21
	500	13	16
Dexamethasone	500	45	57
17-ß-Estradiol	500	53	43
5-α-Dihydrotestosterone	500	40	32
Aldosterone	500	46	35

* Specific [^{3}H]progesterone binding in the two fractions with no steroid additions is expressed as 100 % and these values are 113,000 ± 11,000 and 200,000 ± 17,000 sites/cell for soluble and extract of particulate fraction, respectively.