Characterization and Solubilization of Gonadotropin Receptor of Bovine Corpus Luteum*

(Received for publication, July 6, 1973)

FRANCE HAOUR AND BRH B. SAXENA‡
From the Cornell University Medical College, New York, New York 10021

SUMMARY

Plasma membranes isolated from bovine corpora lutea showed specific binding with $^{125}$I-human chorionic gonadotropin and $^{131}$I-human luteinizing hormone; unlabeled hormones competitively inhibited the binding. The binding of the $^{125}$I-human chorionic gonadotropin to the receptor was a saturable phenomenon and the half-saturation was attained at a concentration of $4 \times 10^{-10}$ M. Maximum hormone receptor binding was obtained within 15 min at pH 7.2 and 37°C. The rate constants of association and dissociation of human chorionic gonadotropin to the receptor determined at 37°C were $2.8 \times 10^{8}$ M$^{-1}$ s$^{-1}$ and $2.1 \times 10^{-5}$ s$^{-1}$, respectively. The dissociation constant calculated from these rates was $8.0 \times 10^{-10}$ M. At 4°C the rate of association was extremely slow and the hormone receptor complex was stable up to 48 hours. The dissociation constants obtained from equilibrium data were calculated to be $1.5 \times 10^{-10}$ M for human chorionic gonadotropin and the number of binding sites were approximately $6.3 \times 10^{10}$ per mg of tissue. The binding of $^{125}$I-human chorionic gonadotropin to the plasma membranes was optimum at pH 7.2 and was reduced at acidic and basic pH. The binding was also inhibited at high concentrations of CaCl$_2$, MgCl$_2$, and NaCl, and in the presence of guanidine HCl and urea. The inhibition of binding at pH 5 and 9 and up to 1 M concentration of various ions was reversible. Treatment of plasma membrane with phospholipase C, neuraminidase, glucosidase, trypsin, and α-chymotrypsin did not decrease the binding of $^{125}$I-human chorionic gonadotropin. Pepsin and phospholipase A inhibited the binding of $^{125}$I-human chorionic gonadotropin to plasma membranes. The plasma membranes were solubilized in detergents. The proteins reprecipitated from the solution by ethanol-guanidine HCl and ion exchange chromatography on DEAE-cellulose in 3 M urea. The receptor activity of the purified fractions could be reconstituted by emulsification with the lipid fraction, indicating the requirement of lipids to retain the native conformation of the receptor. The molecular weight of the active material estimated from Sepharose 4B column in 6 M guanidine HCl and polyacrylamide gel electrophoresis in sodium dodecyl sulfate was between 30,000 to 70,000. This partially purified protein may represent the regulatory subunit of the gonadotropin receptor.

The presence of specific receptors for gonadotropins (FSH, LH, and HCG) in the ovaries (1–12), testes (13–14), and in adrenocortical carcinoma (15) has been demonstrated. The interaction of the gonadotropins to the receptors in the plasma membranes of the intact ovary has been shown to activate adenylate cyclase and that the hormone may enter the cell to stimulate intracellular kinases involved in gonadal steroidogenesis (4, 10, 20). It is not clear whether the receptor and the adenylate cyclase are the components of the same or different molecules. There is evidence for the presence of multiple hormone receptor sites in the plasma membranes of the fat cells which compete for the same adenylate cyclase (21) and that the hormonal binding to the receptor and the activation of adenylate cyclase can occur independently (22–24). It is likely that hormonal action may involve more than one protein molecule. Isolation and characterization of the gonadotropin receptor is therefore of fundamental importance to elucidate the mechanism of action of gonadotropic hormones at the molecular level.

Solubilization of the plasma membrane proteins of the target tissue to isolate the receptors for norepinephrine (22), acetylcholine (25), insulin (26–27), and HCG (28) has been achieved by the use of various ionic and nonionic detergents. In this paper, we describe the characterization and solubilization of receptors for gonadotropins from the plasma membranes of bovine corpora lutea.

* This work was supported by Contract NCIHD-72 2763 and Grants CA-13908 and HD-06543 from the National Institutes of Health and by Grant M72-189 from The Population Council, The Rockefeller University, New York, New York.
‡ Career Scientist Awardee, Contract I-621, Health Research Council of the City of New York.

1 The abbreviations used are: FSH, follicle-stimulating hormone; LH, luteinizing hormone; HCG, human chorionic gonadotropin; SDS, sodium dodecyl sulfate.
MATERIALS AND METHODS

Preparation of Plasma Membranes—Bovine ovaries of early pregnancy (first trimester; fetus of crown to rump length up to 22 cm) were obtained fresh from the slaughter house in chilled containers. The corpora lutea were dissected and either processed for plasma membranes or nitrocellulose filters were prepared. In a typical case, 20 g of the tissues were homogenized by 15 to 20 strokes in a glass-Teflon homogenizer in 200 ml of 10 mM Tris-HCl buffer, pH 7.8, containing 1 mM dithiothreitol and 1 mM MgCl2. The plasma membranes were isolated according to the procedure described in Diagram 1 using the Beckman preparative ultracentrifuge (model L205B; swinging bucket rotor, type SW 25.1). Fractions obtained from the centrifugation and density gradient were examined for purity by electron microscope and for specific binding with 125I-labeled HCG as described below. As shown in Fig. 1, the fractions containing plasma membranes and showing the maximum binding with 125I-HCG were pooled and stored in liquid nitrogen in suitable aliquots. Aliquots of ovarian homogenate and plasma membrane fraction were solubilized in 1 mM NaOH containing 0.1% SDS. The protein content was determined by the method of Lowry et al. (29) using bovine serum albumin as the standard.

Electron Microscopy—An aliquot of the plasma membrane fraction was fixed for 34 hours in 0.25% glutaraldehyde in 0.057 M cacodylate buffer, pH 7.3. The samples were washed for 5 min in chilled 0.25 M cacodylate or phosphate buffer containing 1% Oet, 1.6% sucrose, 4% paraformaldehyde, and 0.2% glutaraldehyde by passing through a graded series of alcohol and embedded in either Epon or Araldite. Thin sections (0.06 to 0.09 μm) were cut and stained in a 4% aqueous uranyl acetate solution and photographed by a Phillips EM-300 electron microscope.

Preparation of Radioactive HCG, LH, and FSH—Radioisotope labeling with 125I was performed essentially by the procedure of Hunter and Greenwood (30). Twenty micrograms of HCG (obtained from Dr. R. F. Canfield, College of Physicians and Surgeons, Columbia University, New York), containing 12,000 i.u. per mg (31) or human LH containing approximately 5,292 i.u. of 2nd IRP human menopausal gonadotropin per mg (32) were dissolved in 20 μl of 10 mM Tris-HCl buffer, pH 7.2, containing 0.9% NaCl in the reaction vial. To the vial, 50 μl of 0.5 M phosphate buffer (pH 7.4), 1.5 μCi of 125I (Cambridge Nuclear, Cambridge, Mass.), and 70 μg of chloramine-T in 20 μl of 0.05 M phosphate buffer, pH 7.4, were added and mixed. After 10 s the reaction was stopped by the addition of 240 μg of sodium metabisulfite in 100 μl of phosphate buffer. Two hundred milligrams of Ioeads (Hyceit Reagents, Houston, Texas) were added to the crude reaction mixture to remove unreacted 125I. The protein mixture was purified by gel filtration through a column of Sephadex G-100 (1 × 30 cm) eluted in and eluted with 0.9% NaCl containing 1% bovine serum albumin. The purity of the labeled hormone was tested by chromatod electrophoresis (34). Precipitation damage and specific activity were determined as described earlier (34). LH, HCG, and LH-8 were also labeled with 131I by lactoperoxidase (35) prepared from milk (R2 = 0.78; Sigma Chemical Co., St. Louis, Mo.). The specific activity of the labeled HCG, LH, and LH-8 were 20 to 35 μCi per μg as determined by trichloroacetic acid precipitation. Five microliters of the crude reaction mixture were diluted to 5 ml with 0.05 M phosphate buffer, pH 7.8, containing 1 mM dithiothreitol and 1 mM MgCl2. The protein content was tested as described above (36). Human pituitary FSH containing 5,677 i.u. of 2nd IRP human menopausal gonadotropin (37) was also labeled with 3H ([3H]KH, 6 Ci per mmole, Amersham-Shell) according to Vitakuitis et al. (38). The biological activity of the [3H]FSH was 3,385 i.u. (50% confidence limits: 2,828 to 3,983 i.u.) as determined by Steelman-Pohley bioassay (39). The specific activity of the [3H]FSH was determined by trichloroacetic acid precipitation as described for LH and HCG.

Binding of Labeled Hormones to Plasma Membrane Receptor—Plasma membrane fractions equivalent to 200 μg of protein were incubated at 37°C for 45 min with 125I-HCG or 125I-LH (1 to 1.5 × 1010; specific activity, 30 to 35 μCi per μg) or with [3H]FSH (1.5 × 1010 μCi per μg) in 300 μl of 10 mM Tris-HCl buffer, pH 7.2, containing 0.1% bovine serum albumin. Similar incubation was performed with an excess of unlabeled LH and HCG (1 × 1012 M) as well as with unlabeled FSH (1 × 109 M). The receptor-bound hormone was separated by filtration through an oxoid membrane of pore size 0.45 μm (Amersham-Sheela). The hormone-receptor complex retained on the filter was washed with 2 to 3 ml of 1% bovine serum albumin in 0.9% NaCl and 1 ml of plasma. Usually less than 1% of the total radioactivity of the labeled hormone was recovered. The filters were transferred into glass vials and the radioactivity was counted in a Packard Auto Gamma counter with an efficiency of 51% for 125I. Reagent blank as well as controls were performed, with the homogenates of rat rectus muscle, with each experiment. The specific binding was defined as the difference between the total binding and the binding in the presence of the excess unlabeled hormones (40).

In the case of [3H]FSH, the filters were solubilized in the glass vials by the addition of 1 μl of TS 1 (Research Product International Corp.) and counted for radioactivity in Packard scintillation counter with an efficiency of 40% (Packard Instrument Co.). Molecular weights of 32,000, 28,000, and 40,000 were used in the calculation of molar uptake of FSH, LH, and HCG, respectively.

Characterization of Plasma-Membrane Receptor—To establish certain presentations, hormones, and ions (sodium, calcium, and magnesium) or hormone-receptor binding was examined (Figs. 2, 3, and 9).

Binding Constants of Hormone-Receptor Interaction (Table I)—Association constant (K), dissociation constant (KD), and K of hormone-receptor binding were determined assuming hormone-receptor binding as an over-all second order reaction. Scatchard analysis (41) of data obtained from saturation curves was used to estimate the number of receptor sites. The specificity of the hormone-receptor binding was established by competition analysis with increasing quantities of the unlabeled hormones (Fig. 7).

Enzymatic Treatment of Plasma Membranes (Table II)—Phospholipase C (Clostridium welchii) and phospholipase A (bee venom) were obtained from Sigma, St. Louis, Mo. Plasma membranes were incubated with phospholipase A and C in 10 mM Tris-HCl buffer, with neuraminidase (Worthington Biochemical Corp., Freehold, N.J.), glucosidase (Almond Emulsin, National Biochemicals Corp., Cleveland, Ohio), papain, trypsin, and α-chymotrypsin (Mann Research Lab., Orangeburg, N.Y.) in 0.1 M NaCl containing 0.01 M NaHCO3 and 1 mM CaCl2 for 1 hour at 37°C. In another experiment, 1 mg of plasma membrane protein was also incubated with 0.1 to 10 μg of phospholipase C under conditions described above (Fig. 10). After incubation, the plasma membranes were washed three times with excess of 10 mM Tris-HCl buffer, pH 7.2, containing 1% bovine serum albumin. Soybean trypsin inhibitor (Sigma, St. Louis, Mo.) in 10-fold excess was added to stop the action of proteolytic enzymes. The hormonal binding of the 200 μg of protein equivalent of treated membrane was tested as described earlier.

Solvilization of Plasma Membranes—Solvilization of the plasma membranes was attempted at 37°C and 4°C in various ionic strengths with 1% sodium deoxycholate (SDS) or 0.1% sodium deoxycholate (SDS) (Pierce Chemicals), as well as non-ionic detergents like Brij 35 (Mann Research Laboratories), Triton X100 (Rohm & Haas), Lubrol WX (General Biochemicals), Igepal 630 (General Aniline and Film Corp.) and Tween 80 (Schwarz-Mann). The solubilized membranes were centrifuged at 100,000 × g in a Beckman ultracentrifuge L205B of rotor type 2 for 1 hour and at 900,000 × g in a Beckman ultracentrifuge L205B of rotor type 30 for 1 hour. The solubilized plasma membrane material was considered as the evidence for the solubilization of most of the plasma membranes. The solubilized membranes were also not retained during filtration on the oxoid filter of pore size 0.45 μm. The solubilized receptor was precipitated at 4°C by making the solution 10% in ammonium acetate (w/v) and 30% NaCl (w/v). The precipitate was washed three times with 10 mM Tris-HCl, pH 7.2. The hormone-binding ability of the precipitate was determined in the same manner as for the intact plasma membranes. The assay of the receptor protein in insolu-
ble form was also applicable to various fractions obtained following the fractionation of the receptor proteins in the presence of various types and concentrations of the solubilizing agents. The plasma membrane proteins were also solubilized in various concentrations of guanidine HCl and urea as shown in Fig. 14. From the data presented in Fig. 12 it was obvious that the plasma membranes were soluble in higher concentrations of guanidine HCl and fractions of Triton X-100, Lubrol WX, sodium deoxycholate, and SDS. However, to avoid the formation of micelles (42) and consequent difficulties in removing the detergents, the plasma membranes were solubilized in 6 M guanidine HCl for further purification on a column of SEpharose 4B.

**Purification of Plasma Membrane Proteins on Sepharose 4B—** Prior to use, guanidine HCl (Heico, Inc., Delaware Water Gap, Pa.) was purified by the addition of 1 g of charcoal (Norit A, Eastman Kodak) to 100 ml of a 6 M guanidine HCl solution. The charcoal was removed by filtration. A column of Sepharose 4B (2.5 × 100 cm) (Pharmacia, Piscataway, N.J.) was equilibrated by the addition of 0.1 M of Tris-HCl buffer, pH 7.2; containing 3 M urea, 1 mM MgCl₂, and SDS. The column was then 4809 for 20 min, centrifugation, 4809 for 20 min, supernatant (S₁). The clear solution was aspirated and concentrated to 5 ml by ultrafiltration (PM-10, Amicon Corp., Lexington, Mass.). The concentrated material was applied to the column of Sepharose 4B. The column was eluted with 6 M guanidine HCl and 2-ml fractions were collected per 15 min. The eluate was divided into six fractions as shown in Fig. 15. Each of these fractions was dialyzed to eliminate the guanidine HCl to recover the receptor protein as a precipitate. These precipitates were tested for hormonal binding. The protein fractions were also emulsified (Bronson sonicator W 180; three pulses of 20 s at 50 watts) with lipid fractions isolated during the solubilization of the plasma membranes with guanidine HCl as well as lipids obtained in the top layer of the sucrose density gradient used for the purification of the plasma membrane protein (to lipid ratio, 5:1, v/v; 1 ml of fraction contained 400 to 500 µg of protein). Phospholipids, such as phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine, in concentrations of 1 to 10 mg per ml, also were emulsified with the protein. Fraction III from the column of Sepharose 4B containing the plasma membranes was usually 70 to 80% of the total binding capacity of the corresponding corpora lutea homogenate, suggesting a small loss of plasma membranes in other subcellular fractions. Fractions 10 to 22 showed maximum hormone-binding activity (Fig. 1) and were used in all subsequent studies. The electron photomicrographs of these fractions showed predominantly plasma membranes. Contamination with mitochondria was in the range of 15 to 20%. Micromolar contamination was insignificant.

**RESULTS**

**Isolation and Characterization of Plasma Membranes of Bovine Corpora Lutea—** Using the procedure described in Diagram 1, 1 g of corpora lutea yielded approximately 7 to 10 mg of plasma membrane protein. The hormonal binding capacity of the purified plasma membranes was usually 70 to 80% of the total binding capacity of the corresponding corpora lutea homogenate, suggesting a small loss of plasma membranes in other subcellular fractions. Fractions 10 to 22 showed maximum hormone-binding activity (Fig. 1) and were used in all subsequent studies. The electron photomicrographs of these samples showed predominantly plasma membranes. Contamination with mitochondria was in the range of 15 to 20%. Micromolar contamination was insignificant.

---

**Polyacrylamide Gel Electrophoresis in SDS—** Plasma membranes, guanidine HCl-soluble material, and various fractions obtained from Sepharose 4B, DEAE-cellulose, and isoelectric focusing columns were analyzed by disc electrophoresis in 6.5% polyacrylamide gels containing 1% SDS according to the method of Fairbanks (44). Protein bands were visualized by staining with Coomassie blue and glycoprotein bands were stained with Schiff’s reagent. Molecular weight of various protein components was estimated from the mobility of proteins of known molecular weights, for example, bovine serum albumin (mol wt 69,000), egg ovalbumin (mol wt 45,000), and lysozyme (mol wt 14,300), determined simultaneously with those of the receptor protein fractions.

**Diagram 1. Preparation of plasma membrane from bovine corpora lutea.**

**Fig. 1. Specific binding of various fractions to ¹²⁵I-HCG from bovine corpora lutea by continuous sucrose density gradient.**
TABLE I

Equilibrium and kinetic parameters of binding of $^{125}$I-HCG, $^{125}$I-LH, and $[^3H]$FSH to plasma membranes of bovine corpora lutea at $37^\circ$

<table>
<thead>
<tr>
<th></th>
<th>Association ($K_a$)</th>
<th>Dissociation ($K_d$)</th>
<th>$K_a/K_d$</th>
<th>Equilibrium$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCG</td>
<td>$2.8 \times 10^6$</td>
<td>$2.1 \times 10^-8$</td>
<td>$8.0 \times 10^-10$</td>
<td>$1.5 \times 10^-10$</td>
</tr>
<tr>
<td>LH</td>
<td>$1.4 \times 10^-9$</td>
<td>$0.0 \times 10^-9$</td>
<td>$1.4 \times 10^-10$</td>
<td>$0.0 \times 10^-9$</td>
</tr>
<tr>
<td>FSH</td>
<td>$9.0 \times 10^-9$</td>
<td>$0.0 \times 10^-9$</td>
<td>$9.0 \times 10^-9$</td>
<td>$0.0 \times 10^-9$</td>
</tr>
</tbody>
</table>

$^a$ Apparent $K$ calculated from saturation.

Fig. 2. Binding of $^{125}$I-HCG to plasma membranes at various pH values. Plasma membranes (200 μg of protein) were incubated with (0.5 × 10^-8 M) $^{125}$I-HCG in 10 mM Tris-HCl buffer containing 0.1% bovine serum albumin.

Fig. 3. Binding of $^{125}$I-HCG to plasma membranes of bovine corpora lutea as a function of time and temperature. Plasma membranes (200 μg of protein) were incubated with (0.5 × 10^-8 M) $^{125}$I-HCG in 10 mM Tris-HCl buffer, pH 7.2, containing 0.1% bovine serum albumin at 37 and $4^\circ$. Specific binding at $4^\circ$ (△) and at 37$^\circ$ (O) was maximum. Nonspecific binding in the presence of (1 × 10^-7 M) HCG at $4^\circ$ (△) and at 37$^\circ$ (O) was increased.

Binding of Labeled Gonadotropins to Plasma Membrane—$^{125}$I-HCG and $^{125}$I-LH retained 80 to 90% and $[^3H]$FSH retained approximately 60% of the biological activity, which is in agreement with the values reported by Vaitukaitis et al. (38) and Miyachi et al. (35). Iodination of LH and HCG by the chloramine T or lactoperoxidase method gave similar results when specific activities were lower than 30 μCi per μg. The binding capacity of the plasma membrane was pH-dependent. As shown in Fig. 2, the binding was maximum at pH 7.2 and decreased at lower or higher pH values. The decrease in the binding capacity observed at pH 6 or 9 was, however, reversible since washing and resuspension of the membrane in Tris-HCl buffer, pH 7.2, restored the binding capacity to normal levels.

The binding of $^{125}$I-HCG to the plasma membrane was a rapid process. At 37$^\circ$ most of the binding was obtained within 15 min (Fig. 3). A slow increase in the nonspecific binding occurred during the next 2 hours. Partial destruction or modification of the plasma membrane structure could explain the slow increase in the nonspecific binding during prolonged in vitro incubation at 37$^\circ$. $K_a$ calculated at 37$^\circ$ from the data in Fig. 3 was $2.8 \times 10^{6}$ M$^{-1}$ s$^{-1}$ (Table I). At 37$^\circ$ dissociation of hormone-receptor complex was very rapid (Fig. 4) and $K_d$ was calculated to be $2.1 \times 10^{-5}$ s$^{-1}$ (Table I). The hormone-receptor binding was slow at 4$^\circ$ (Fig. 3) and equilibrium was not reached for several hours. The dissociation of the hormone receptor complex at 4$^\circ$ was extremely slow (Fig. 4) even in the presence of an excess of unlabeled hormone ($10^{-7}$ M). The hormone-receptor complex was stable for more than 48 hours at 4$^\circ$.

The specificity of the $^{125}$I-HCG binding to the plasma membrane was illustrated by the competitive inhibition of the binding of $^{125}$I-HCG by unlabeled HCG at 37$^\circ$ (Fig. 5). A total inhibition of the binding was obtained by concentrations of the unlabeled HCG greater than $2 \times 10^{-8}$ M and a 50% inhibition was attained at a concentration of 15 ng of HCG per ml or $4 \times 10^{-10}$ M. Human LH also inhibited the binding of $^{125}$I-HCG to the plasma membranes, but FSH, growth hormone, and prolactin up to concentrations of 1 μg per ml did not. The α and β subunits, FSH, LH, and HCG did not compete with $^{125}$I-HCG for binding to the plasma membrane receptor, suggesting the need of intact hormone molecule for binding to the receptor.
FIG. 5. Competitive inhibition by unlabeled HCG on the binding of 125I-HCG to plasma membranes of bovine corpora lutea. Incubates containing 250 μg of plasma membrane protein were incubated with 1 × 10^{-10} M 125I-HCG in the presence of increasing amounts of unlabeled HCG.

Characterization of Binding of 125I-HCG, 125I-LH, and [3H]FSH to Plasma Membranes—The binding of the hormone to the receptor was a saturable phenomenon (Fig. 6). With increasing concentrations of 125I-HCG, the saturation was attained between 10^{-9} to 10^{-8} M. Scatchard plot of the equilibrium data indicated a K_d of 1.5 × 10^{-10} M (Fig. 7 and Table 1). The X intercept of the Scatchard plot yielded an estimate of the number of binding sites to be in the order of 2.5 to 10 × 10^{13} moles per mg of plasma membrane protein, which are 100-fold greater than the number of sites in the fresh corpora lutea homogenate (2.5 to 10 × 10^{12} moles per mg of tissue).

Biologically active [3H]FSH bound specifically to plasma membrane of cow corpus luteum (Fig. 8), however, very high nonspecific binding was noticed. The binding capacity of membrane derived from the equilibrium data was evaluated to a value of 1 × 10^{-13} moles per mg of protein. The value of K_d derived from this data is 9.0 × 10^{-9} M (Table 1).

Effect of Various Treatments on Plasma Membranes—Sonication did not destroy the ability of the purified plasma membrane to bind 125I-HCG. The competitive inhibition curve with unlabeled HCG was identical to that of the intact membranes. However, the sonicated plasma membranes showed a greater tendency to aggregate, which may explain a slight decrease in the binding capacity of the preparation.

As shown in Fig. 9, an increase in the binding was observed with concentrations of CaCl_2 up to 10 mM. Such an effect was not seen in the case of MgCl_2, perhaps due to the fact that membranes were prepared in Tris-HCl buffer already containing 10 mM MgCl_2 and the incubation medium also contained 0.2 to 0.5 mM MgCl_2. An increase in the concentrations of various ions, however, inhibited the binding of 125I-HCG to the receptor. In the case of NaCl, a 50% inhibition in the binding was observed at a concentration of 0.35 M. A similar inhibition in the binding of the hormone to sonicated plasma membranes was observed at NaCl concentrations greater than 1 M. In the case of CaCl_2 and MgCl_2, 50% inhibition was observed at a concentration of 0.12 M. It is interesting to note that the inhibition of the binding at salt concentrations up to 1 M was fully reversible after washing and resuspension of the plasma membranes in the incubation buffer. These observations indicate that low concentrations of divalent ions like Mg^{2+} and Ca^{2+} facilitate the binding.

Phospholipase A suppressed the ability of the intact and sonicated plasma membranes to bind with 125I-HCG, whereas phos-
Fig. 8. Binding of \(^{3}H\)-FSH to plasma membrane of bovine corpora lutea. Plasma membranes (200 \(\mu\)g of protein) were incubated with increasing concentrations of \(^{3}H\)-FSH in 300 \(\mu\)l of 10 mM Tris-HCl buffer, pH 7.2, containing 0.1% bovine serum albumin at 37°C for 45 min. Total binding, O--O; nonspecific binding in the presence of \(1 \times 10^{-5}\) M FSH, O--O; blank or radioactivity adsorbed on the oxoid filter, △--△.

Fig. 9. Effect of various ionic concentrations on the binding of \(^{125}I\)-HCG to plasma membranes of bovine corpora lutea. Plasma membrane (200 \(\mu\)g of protein) were incubated with increasing concentrations of \(^{125}I\)-HCG in 10 mM Tris-HCl buffer, pH 7.2, containing 0.1% bovine serum albumin at 37°C for 45 min. NaCl, \(-\cdot\cdot\cdot\cdot\cdot\); MgCl\(_2\), O--O; CaCl\(_2\), △--△; and control, △--△.

Phospholipase C significantly increased the binding ability. As shown in Table II and Fig. 10, the binding ability of the sonicated plasma membranes was increased 2.4-fold by treatment with phospholipase C; however, at higher concentrations the binding ability was inhibited. It is likely that at lower concentrations phospholipase C deaggregated the plasma membrane to unmask additional receptor sites, whereas at higher concentrations of phospholipase C, the plasma membrane structure was disrupted. As shown in Table II, hormone-binding ability of the plasma membranes was stable to neuraminidase; however, glucosidase treatment increased the binding of \(^{125}I\)-HCG. Trypsin and \(\alpha\)-chymotrypsin did not affect, whereas pepsin completely suppressed, the binding of the hormone to the plasma membranes. It may be suggested that the complex lipid-protein nature of the plasma membrane receptor may be a steric hindrance for the enzymes to attack specific sites.

Solubilization of Plasma Membranes—Ionic detergents (deoxycholate, SDS) and nonionic detergents ( Triton X-100, Lubrol WX, Igepal CO-630, Brij 35, and Tween 80) have been used to solubilize plasma membranes. As shown in Fig. 11, the concentration of the detergent necessary to solubilize the membrane receptor is temperature-dependent. Attempts to solubilize the receptor at room temperature or at 37°C adversely affected the hormone-binding ability of the receptor. Solubilization was therefore performed at 4°C. At this temperature more than 10-fold detergent was required to totally solubilize 1 mg of purified plasma membrane. Effectiveness of various detergents, as measured by their ability to decrease the activity of labeled plasma membranes retained on the oxoid filter, was in the fol-

**Table II**  
Effect of enzymatic treatment on plasma membranes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific binding</th>
<th>Incubation pH</th>
<th>E:S</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-Chymotrypsin</td>
<td>109 (100-117)</td>
<td>8</td>
<td>1:60</td>
</tr>
<tr>
<td>Trypsin</td>
<td>95 (86-100)</td>
<td>8</td>
<td>1:30</td>
</tr>
<tr>
<td>Pepsin</td>
<td>3 (0-8)</td>
<td>5</td>
<td>1:60</td>
</tr>
<tr>
<td>Emulsin</td>
<td>205 (193-239)</td>
<td>5</td>
<td>1:3</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>77 (63-90)</td>
<td>5</td>
<td>1:30</td>
</tr>
<tr>
<td>Phospholipase A</td>
<td>10 (5-12)</td>
<td>7.2</td>
<td>1:10</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>112 (100-119)</td>
<td>7.2</td>
<td>1:10</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>240 (230-258)</td>
<td>7.2</td>
<td>1:10</td>
</tr>
</tbody>
</table>

* Average and range of the triplicate determination.
* Protein.
* Sonicated plasma membranes.
FIG. 11. Solubilization of plasma membranes of bovine corpora lutea labeled with $^{125}\text{I}}$-HCG. Plasma membranes (200 μg of protein) were incubated at 37°C for 45 min. The detergents were added to the medium. After 1 hour at 4°C or at 37°C, the incubates were filtered through oxoid filters. The oxoid filters were counted for radioactivity.

Fig. 12. Solubilization of plasma membranes of bovine corpora lutea labeled with $^{125}\text{I}}$-HCG and treated as described in Fig. 11. DOC, deoxycholate.

After solubilization and centrifugation at 100,000 × g for 1 hour or at 300,000 × g for 2 hours, the soluble membrane protein was precipitated by making the solution 10% in ammonium acetate and 30% in ethanol. After centrifugation the precipitates were washed with Tris-HCl buffer and the binding ability of the precipitates was tested. The precipitates obtained from plasma membranes solubilized in Triton X-100, Igepal 630, and Brij 35 showed significant recovery of the binding activity. These experiments indicated that nonionic detergents like Brij 35, Triton X-100, and Igepal 630 are promising media for the solubilization of the plasma membranes and purification of the receptor protein. The precipitates recovered from the plasma membranes solubilized in Brij 35 showed the highest specific binding ability of approximately 30% of the initial binding activity of the intact plasma membranes.

Solubilization by Guanidine HCl and Protein Fractionation—Our studies suggested that the presence of 0.2 M guanidine HCl and 0.75 M urea caused a 50% inhibition of the hormone-receptor binding (Fig. 13). It is of interest that urea or guanidine HCl concentrations up to 1 M failed to dissociate the hormone-receptor complex. Further increase in the concentration of urea or guanidine HCl resulted in the disruption or solubilization of the plasma membranes (Fig. 14).

The plasma membrane proteins solubilized in 6 M guanidine HCl were purified on a column of Sepharose 4B. As shown in Fig. 15, Fraction I eluted with the void volume contained pre-
Characterization of the lipid fraction should permit the identification of the essential lipid involved in the receptor structure.

Ion exchange chromatography of Fraction III on DEAE-cellulose resulted in further purification of the receptor protein. The fraction eluted between 0.04 M and 0.2 M NaCl, as indicated by the hatched area in Fig. 16, showed specific binding with \(^{125}\)I-HCG after reconstitution with the lipids; however, this fraction was not homogeneous when examined by polyacrylamide gel electrophoresis in SDS. Efforts toward further purification of the receptor protein by isoelectric focusing in 2 M urea resulted in the separation of several protein bands. Due to the precipitation of these bands on the column, a discrete isolation of the protein associated with the receptor has not been achieved. The isoelectric point of the fraction containing receptor activity was approximately 4.5. On the basis of the recovery of the protein, it was estimated that at least 1000 fold more purification was necessary to obtain a homogeneous protein with receptor activity.

**Polyacrylamide Gel Electrophoresis in SDS**—Disc electrophoretic pattern of intact plasma membrane (Fig. 17) indicated the presence of at least 20 major protein bands, whereas Schiff’s reagent indicated the presence of four glycoproteins in the molecular weight range of 100,000, 80,000, 40,000, and 10,000. From the Sepharose column, Fraction I contained highly aggregated material which did not enter the gel. Fractions II, IV, and V showed multiple protein bands in the molecular weight range 10,000 to 100,000. A little receptor activity present in these fractions was due to overlap with Fraction III which contained most of the receptor protein activity. Fraction III showed protein bands of molecular weight range between 30,000 and 70,000. This estimate was close to the molecular weight of Fraction III estimated from the Sepharose 4B column.

**DISCUSSION**

The general characteristics of the binding of \(^{125}\)I-HCG to the plasma membranes of bovine corpora lutea are comparable with those reported for gonadal tissue. Most of the specific and high affinity binding was demonstrated by the plasma membranes. The binding phenomenon was pH- and temperature-dependent and indicated the requirement of divalent ions (Mg\(^{2+}\) or Ca\(^{2+}\)) in the incubation medium for optimum binding. The estimates of \(K_d\) of the binding of \(^{125}\)I-HCG to the plasma membranes calculated from equilibrium data at 37\(^\circ\) and from the ratio of rate constants were 8.0 and 1.5 \(\times 10^{-12}\) M as compared to 3 and 1.1 \(\times 10^{-4}\) M reported for the binding of bovine LH to plasma membranes of bovine corpus luteum (11). These values can also be compared with those obtained for the plasma membrane of rat superovulated ovaries (3 \(\times 10^{-12}\) M (4)), for rat testes homogenate (3 \(\times 10^{-12}\) M (44)), and for human corpus luteum (8.6 \(\times 10^{-12}\) M (45)). Ovarian slices yielded a lower value of 1.5 \(\times 10^{-8}\) M (8). It seems that the apparent affinity of the hormone binding increases in purified plasma membranes. The lower \(K_d\) in crude receptor preparations may partly be due to nonspecific adsorption or enzymatic destruction of the hormone during incubation. It is interesting to note that IICG shows similar binding ability for the receptor sites of rat testis, superovulated rat ovary, or pregnant cow and human corpora lutea, suggesting lack of species specificity of hormone-receptor binding; the possibility of subtle quantitative differences, however, are not ruled out. The number of binding sites per mg of fresh bovine ovarian tissue was estimated to be 5 \(\times 10^{-14}\) M, a value which is close to the number reported for LH in human corpus luteum (1.8 \(\times 10^{-14}\) M (8)) and for HCG in superovulated rat ovaries (3 \(\times 10^{-12}\) M (8)).
FIG. 16. Disc electrophoresis in 6.6% polyacrylamide gel in 1% SDS. Two hundred micrograms of proteins of plasma membranes or fractions from the column of Sepharose 4B (Fig. 15) were analyzed. The gels were stained with Coomassie blue (CB) or with Schiff’s reagent (PAS). On the left are shown the molarities of proteins of known molecular weights (see text) analyzed simultaneously.

FIG. 17. Fractionation of plasma membrane protein (Fraction III from Sepharose 4B column, Fig. 15) on DEAE-cellulose.

ovary \((1.8 \times 10^{14} \text{ M} (4))\). It is interesting to note that the affinity of the gonadotropin receptor for HCG was comparable to the values obtained in the case of insulin-receptor interaction in the fat cells (27-47). The weight of receptor present per mg of membrane protein can be calculated from the number of binding sites \((6.3 \times 10^{13} \text{ moles per mg})\) and from a postulated molecular weight of the receptor protein of 70,000. Thus, approximately 90 ng of receptor protein are present in 1 mg of plasma membrane proteins. Hence, approximately 20,000-fold purification of the purified plasma membranes was necessary to obtain chemically pure receptor.

At low temperature (4°), the hormone-receptor complex was stable for more than 48 hours, even in the presence of excess of unlabeled HCG which is in agreement with similar observations obtained with rat ovarian membranes (4), homogenates (48), and intact granulosa cells (49). It is logical to assume that varying amounts of endogenous LH may remain bound to the receptor in plasma membranes prepared from the ovaries at different stages of the reproductive cycle. Further support of this conjecture may be derived from the fact that the number of receptor sites to human LH in human ovary vary at various states of the reproductive (50) and menstrual cycle (9). Channing and Kammerman (49) have recently suggested that the endogenous hormone bound to the ovarian receptor may interfere in the measurement of the binding of ^{125}\text{I}-HCG to the ovarian cells. It may be mentioned that our preliminary experiments
have revealed that the number of binding sites actually increased in the plasma membranes after the dissociation of endogenously bound hormone under suitable conditions of pH, temperature, and the composition of the incubation mixture. The dissociated hormone exhibited immunological identity with the native hormone in the radioimmunoassay. The details of these experiments will be published elsewhere. The high variability observed in the determination of the number of hormone binding sites in the target organs may also be due to the ratio of high and low affinity receptors (50). One would therefore invoke a concept of apparent and total binding sites in the evaluation of hormone receptor binding phenomenon.

The results obtained in our studies have indicated that divalent ions, at low concentrations, facilitate the binding of HCG to plasma membranes. However, concentrations greater than 10 mm CaCl₂ and MgCl₂ produced an inhibiting effect similar to the inhibition of glucose binding to fat cells (47) and thyroid-stimulating hormone binding to thyroid plasma membranes (51). These observations are in variance with those obtained for insulin binding to the liver and fat cells (27) and for HCG binding to the plasma membranes of superovulated rat oocytes (4). In these studies, an increase in the binding ability of the plasma membrane with increasing NaCl concentration up to 2 M was reported. It is interesting to note that the suppression of binding caused by concentrations of NaCl, MgCl₂, and CaCl₂ up to 1 M was fully reversible, suggesting that at these concentrations, no irreversible conformational changes occurred in the plasma membrane structure. The supernatants recovered following treatment with higher salt concentration (greater than 1 M) showed a precipitate after the removal of salts by dialysis which contained specific binding with [3H]-HCG suggesting that some receptor was solubilized and may account for the loss of binding activity at higher salt concentrations.

Similarly, the inhibition of the binding caused by urea or guanidine HCl up to the concentrations of 1 M was fully reversible, indicating no significant destruction of the receptor (Fig. 13). The stability of the hormone-receptor complex was demonstrated when concentrations up to 1 M urea or guanidine HCl failed to dissociate the hormone-receptor complex (Fig. 14). These observations confirm the high stability of the noncovalent linkage between hormone and receptor molecules. The protein nature of the plasma membrane receptors has been amply demonstrated (47, 52). In view of the highly hydrophobic nature of the plasma membrane structure, the results of the proteolytic attack should be interpreted with caution. Our results indicate that the receptor was stable to the proteolytic enzymes like trypsin and chymotrypsin due to steric hindrance for these enzymes to reach the specific peptide linkages; however, pepsin treatment at pH 5.0 destroyed the receptor activity. Neuraminidase and glucose oxidase also do not suppress the binding ability, indicating that, if any, a minor carbohydrate moiety might be involved in the receptor structure. An increase in the hormone-binding ability following treatment with phospholipase in the case of insulin receptor (17) and HCG receptor (4) has been reported. In the case of glucose receptor there was a decrease in the binding (62). In the case of intact plasma membranes, our studies did not show any significant increase in the binding. A very consistent increase, however, was observed in the case of sonicated membranes. These considerations are important for the explanation of the general physiological role of receptor sites. If the number of receptor sites can be modified under various pathological or physiological (9, 46) conditions, one may speculate that either existing receptors are unmasked or that new receptors are inserted in the membrane structure. Evidence of a change in the number of binding sites has been obtained in the case of insulin receptors (46). Our data would support the hypothesis that there are no hidden binding sites in the case of HCG receptor and that the apparent change in the number of sites may be a result of the degree of binding sites occupied by the endogenous hormone.

Tritiated FSH was specifically concentrated by the ovary of immature or pseudo-pregnant rat (28). Our data confirms the presence of a specific binding site for FSH in the plasma membranes of the corpus luteum of pregnant cow. The Kd of the binding was estimated to be 0.0 × 10⁻⁵ M and can be compared to the value of 7 × 10⁻⁵ M reported in the case of rat testis (14). Since FSH did not compete with [3H]-HCG for binding, it may be postulated that the FSH is bound to the sites distinct from those for HCG.

Solubilization of the plasma membranes at temperatures 28° and 37° did not result in an active form of the receptor. This is in contrast to the insulin receptor. Possibly at higher temperature there was a greater proteolytic destruction. Solubilization of the receptor by guanidine HCl and the recovery of binding activity of the protein moiety after reconstitution with the lipid fraction suggested that the receptor proteins were not irreversibly denatured by guanidine HCl and that the reconstitution of the native structure requires lipids. It is of interest to note that dissociation of LH into subunits by guanidine HCl and their reconstitution after removal of guanidine HCl has been achieved (53). Denaturation of human serum albumin by the same chaotropic agent has been shown to be a reversible phenomenon (54). Separation of membrane proteins has been successfully achieved in the presence of guanidine HCl. It has also been demonstrated that the phospholipids were able to restore hormone stimulatory activity of cat myocardial adenylate cyclase (22) as well as glucose 6-phosphatase activity of the liver microsomes (56). The data presented here showed that the gonadotropin receptor could be separated into protein and lipid moieties by solubilization in guanidine HCl and that the receptor could be reconstituted in vitro by combining the lipid and protein moieties. The reconstituted receptor exhibited restoration of the specific binding with [3H]-HCG.

The molecular weight of the protein moiety of the receptor determined in the presence of 6 M guanidine HCl and SDS was in the range of 30,000 to 70,000. These estimates are lower than the values obtained in detergents for the intact receptor protein for insulin (300,000) (27) or for acetylcholine (360,000) (25). The cholenergic receptor has also been reported to be constituted of subunits of the molecular weight of 40,000 to 50,000 and a β adrenergic receptor was recently described in two molecular forms of molecular weights 40,000 and 100,000 (57). According to the concept of Sutherland et al. (55) a hormone receptor may involve a regulatory subunit, a catalytic subunit (adenylate cyclase), and, perhaps, intermediary couplers. The partially purified protein moiety of a molecular weight between 30,000 to 70,000 (57). According to the concept of Sutherland et al. (55) a hormone receptor may involve a regulatory subunit, a catalytic subunit (adenylate cyclase), and, perhaps, intermediary couplers. The partially purified protein moiety of a molecular weight between 30,000 to 70,000 was described here may represent the regulatory subunit of the gonadotropin receptor involved in the recognition of the hormone. It is also concluded that the lipid moiety is essential for the native conformation of the receptor structure. Further characterization of the protein and lipid moieties of the receptor, which appears promising by using detergents and guanidine HCl as solubilization agents, would permit a precise elucidation of the structure-function relationship of the gonadotropin receptor.

Acknowledgments—We are grateful to Drs. R. Canfield and

Downloaded from http://www.jbc.org/ by guest on August 15, 2017
O. P. Bahl for HCG and their subunits, to Dr. P. Rathnam for human pituitary FSH, LH, and their subunits, to Dr. E. Nunez for electron microscopic studies, to Ms. Nancy Moore for technical assistance, and to Ms. Sharon Wardlaw for excellent help during the course of these studies.

Addendum—Since this manuscript was submitted for publication, Gospodarowicz (50) has described the binding of bovine LH to bovine corpora lutea and determined a $K_d$ of $3 \times 10^{-8}$ M.

REFERENCES

2. RAJANENI, H., AND VANKA-PERTTILA, T. (1972) Endocrinology 90, 1
3. KAMMERMAN, S., AND CANFIELD, R. E. (1972) Endocrinology 90, 384
7. TSURUHARA, T., VAN HALL, E. V., DUFU, M. L., AND CATT, K. J. (1972) Endocrinology 91, 463
38. LEIDENBERGER, F., AND REICHERT, L. E., JR. (1972) Endocrinology 91, 135
40. DWIGGINS, C. W., JR., BOLLEN, R. J., AND DUNNING, H. N. (1960) J. Physiol. Chem. 34, 1175
42. FAIRBANKS, G., STEG, T. L., AND WALLACH, D. F. H. (1971) Biochemistry 10, 2606
44. KAHN, C. R., NEVILLE, D. M., JR., AND ROTH, J. (1973) J. Biol. Chem. 248, 244
45. CUATRECASAS, P. (1971) J. Biol. Chem. 246, 7265
47. CHANNING, C. P., AND KAMMERMAN, S. (1962) Endocrinology 72, 531
53. CARRAWAY, T. F., JR., KOHN, L. E., JR. (1972) J. Biol. Chem. 246, 4092
Characterization and Solubilization of Gonadotropin Receptor of Bovine Corpus Luteum
France Haour and Brij B. Saxena


Access the most updated version of this article at http://www.jbc.org/content/249/7/2195

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/249/7/2195.full.html#ref-list-1