Properties of the Luteinizing Hormone Receptor of Isolated Bovine Corpus Luteum Plasma Membranes*

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SUMMARY

Luteinizing hormone binding sites with high affinity and specificity for ovine-luteinizing hormone have been shown to be present in a purified plasma membrane preparation obtained from bovine corpus luteum. The specific binding of $^{125}$I-luteinizing hormone to the membranes is a saturable process with respect to $^{125}$I-luteinizing hormone. Native luteinizing hormone competes for the binding in a way expected from the biological identity of the 2 molecules. Human chorionic gonadotropin and pregnant mare serum gonadotropin, two gonadotropins which have luteinizing hormone activity, compete for the binding site of luteinizing hormone, with an affinity which is less than that of native luteinizing hormone. The same holds for the $\alpha$ and $\beta$ chains of luteinizing hormone which showed, respectively, 100 and 200 times less affinity than luteinizing hormone for its binding site. Follicle-stimulating hormone, which does not have intrinsic luteinizing hormone activity, does not compete for the binding site of luteinizing hormone to an extent greater than its contamination by luteinizing hormone allows.

The binding of $^{125}$I-luteinizing hormone is temperature-dependent and reaches its maximum in 10 min at 37°. The rate constant of the luteinizing hormone-membrane association ($2.17 \times 10^6$ M$^{-1}$ s$^{-1}$) and dissociation ($2.46 \times 10^{-3}$ s$^{-1}$) have been measured independently at 23 and 10°. The dissociation constant (1.13 $\times 10^{-9}$ M) based on these rate constants is similar to that (3 $\times 10^{-9}$ M) calculated separately from equilibrium data. Measurement of the rate constants at various temperatures gives similar values for the dissociation constant. This shows that the decrease in dissociation rate is proportionately the same as the decrease in association rate. Binding is maximal at pH 7.6 and is not affected by Ca$^{2+}$ concentration in the range of 0.1 to 20 mM. The effects of different enzymatic preparations on the binding site of luteinizing hormone have been investigated. It is not affected by DNase, trypsin, chymotrypsin, pepsin, and collagenase. Treatment of the membrane preparations by neuraminidase increased the binding capacity for luteinizing hormone by 2-fold. Phospholipase C, as well as phospholipase A, decreases it by half.

Study of the specific binding of $^{125}$I-luteinizing hormone to cubecellar fractions of bovine corpus luteum has shown that the specific binding of luteinizing hormone is localized to the plasma membrane fraction (1). Since the disruption of cellular structures leads to the loss of biological activity, the interaction of LH with its receptor site can be best defined by looking at the binding of $^{125}$I-LH to the membranes. One should then make certain that this interaction is specific and that $^{125}$I-LH does not bind to structures other than its receptor site. The present report presents detailed data on the properties of the binding interaction between bovine corpus luteum plasma membrane and $^{125}$I-LH. The kinetic data for binding of $^{125}$I-LH to its receptor site will be further utilized for the isolation of the LH receptor site from plasma membrane of bovine corpus luteum.

EXPERIMENTAL PROCEDURE

Materials—Highly purified ovine LH is prepared by the method of Papkoff et al. (2) and is further purified by chromatography on diethylaminoethyl cellulose to remove contaminating thyroid-stimulating hormone as described by Pierce and Carsten (3). The biological activity measured by the ovarian ascorbic acid depletion assay is 2.75 units per mg (95% confidence limits 2.1 to 3.7) referred to the standard NIH-LH-S17. Thyroid-stimulating hormone activity amounts to 15 to 30 milliunits per mg. Contamination by growth hormone, follicle-stimulating hormone, adrenocorticotropic hormone, or melanocyte-stimulating hormone cannot be detected. Highly purified PMSG (15,000 i.u. per mg) is prepared by the method of Gospodarowicz and Papkoff (4) and Gospodarowicz (5). The isolation of the two subunits of ovine LH is affected in this laboratory by counter current distribution (12 transfers repeated twice) as described by Papkoff and Samy (6) and as modified by Reichert et al. (7).

Amino acid and carbohydrate analyses show the purity of the LH $\alpha$ and LH $\beta$ subunits (6) which is further confirmed by double diffusion in agar, immunoelectrophoresis, quantitative precipitation curves, steroidogenic activity (8), and physicochemical properties (9). Highly purified FSH is prepared by the method of Papkoff et al. (10). Its activity is 30 times the standard NIH-FSH-S1. Residual LH contamination is eliminated by chromatography on an anti-LH IgG Sepharose column, and amounts to 0.005% as determined by radioimmunoassay (11).

The abbreviations used are: LH, luteinizing hormone; PMSG, pregnant mare serum gonadotropin; FSH, follicle-stimulating hormone; HCG, human chorionic gonadotropin.

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HCG (2500 units per mg) is obtained from Mann Research Laboratories, New York. Bovine serum albumin Fraction V is from Riker’s Chemical Co., Chicago, Ill. Lactoperoxidase grade B is from Calbiochem, San Diego, Calif. 125I Na, carrier-free, is obtained from New England Nuclear, Boston, Mass.

Neuraminidase (Clostridium perfringens sp., specific activity 3.6), soybean trypsin inhibitor, trypsin, chymotrypsin, collagenase (1.5 units per mg), peptisin, and DNase are obtained from Worthington Biochemical Co., Freehold, N. J. Phospholipase A (from Vipera russelli and bee venom), hyaluronidase (type II), protease (Streptomyces griseus type VI) are from Sigma Chemical Co., St. Louis, Mo. Phospholipase C (15 units per mg) is from Nutritional Biochemicals Co., Cleveland, Ohio. Cellulose acetate filters EGWP (0.2500, 0.2 μ) are obtained from Millipore Co., Bedford, Mass.

Iodination Procedure—The iodination of LH is performed using lactoperoxidase as the catalytic agent. All reactions are carried out in small glass tubes at room temperature. The reagents are mixed continuously with a small magnetic stirrer. In order to minimize the introduction of more than 1 atom of iodine per LH molecule, iodination is performed with an equimolar ratio of 125I to LH. To minimize the deleterious effect of the oxidizing agent, hydrogen peroxide, it is added in approximately equimolar amounts with respect to the concentration of iodine and LH. To obtain large quantities of the iodinated LH for chemical and biological analysis, milligrams of purified LH are iodinated with 125I to which is added a trace of 131I. When low specific activity iodinated LH is needed, the reagents are added rapidly in the following order and amounts: (a) 250 μCi of 131I Na in 5 μl of 0.1 N NaOH; (b) 88 ng of 125I Na in 100 μl of 0.1 N NaOH; (c) 50 μl of 1 M potassium phosphate buffer, pH 7.3; (d) 120 μg of LH in 50 μl of 0.25 M potassium phosphate buffer, pH 7.3; (e) 5 μg of lactoperoxidase in 5 μl of the same buffer. The reaction is initiated by adding 70 ng of hydrogen peroxide in 10 μl of water. To sustain the reaction, 70 ng of hydrogen peroxide are added four times at intervals of 2 min. At 1-min intervals starting at zero time, 5 μl of the iodinated solution are withdrawn, mixed with 0.1 ml of 0.1% bovine serum albumin (w/v), and precipitated with 10% trichloroacetic acid. Precipitates are washed twice with cold 10% trichloroacetic acid, dissolved in 0.2 ml of 0.5 N KOH, and their radioactivity determined in a liquid scintillation counter (Nuclear Chicago model Unilux II). Usually in 10 min, 90 to 95% of the 125I is trichloroacetic acid-precipitable. When iodinated LH of high specific activity is needed, no 131I was added, and 10 mCi of 125I Na (588 ng) are added. The ratios of 125I to LH, lactoperoxidase, and hydrogen peroxide are kept equimolar. After the reaction is completed, final purification is achieved by gel filtration of the iodination solution on columns of Sephadex G-200 equilibrated with 0.9% NaCl, 0.01 M potassium phosphate, pH 7.3.

Integrity of the LH is analyzed by polyacrylamide gel electrophoresis (Fig. 1A). One peak of radioactivity is observed, coinciding with the α subunit of the hormone. Countercurrent distribution of the subunits of LH confirms this observation. All the radioactivity is confined to the α subunit. This suggests that the tyrosyl residue present in the β subunit is not exposed when the LH has its native configuration. The lack of 131I in the β subunit also shows that the reaction is highly specific for tyrosyl residues since no other amino acids are labeled.

The integrity of the antigenic site of 125I-LH is confirmed by a quantitative precipitin test (Fig. 1B). The equivalence zone for LH is the same as that of 125I-LH, and over 90% of the 125I-LH is precipitated. When 131I-LH is applied on an anti-LH IgG Sepharose column (11), 93% is retained on the column, thus proving that the antigenic sites of LH are intact (Fig. 1C). Ninety per cent of the adsorbed 125I-LH is eluted with 6 M guanidine HCl, pH 1.5. The unadsorbed fraction may represent either iodinated peroxidase or denatured LH. When the biological activities of LH and 125I-LH are compared by the ovarian ascorbic acid depletion assay, their activities are indistinguishable (Table I). Their ability to stimulate progesterone biosynthesis (Fig. 2A), as well as release of prostaglandins in vitro from bovine luteal cell suspension (Fig. 2B), is also identical over a 1000-fold range of concentration.

These different criteria indicate that iodination of bovine LH using the lactoperoxidase as a catalytic agent produces no noticeable denaturation of the hormone.

The final activity of ovine LH is 1800 Ci per mm, corresponding to an average incorporation of 1 atom of 131I per molecule of LH. With 90% incorporation of 125I into LH, no further purification of LH by ion exchange chromatography is required to separate LH from 131I-LH.

By increasing the concentration of 131I, an average of more than 1 atom of 131I can be incorporated by a molecule of LH. Since a decrease in biological activity of ovine LH is observed when more than 2 atoms of iodine are incorporated into each molecule

![Fig. 1. A, fractionation of a 12% acrylamide gel pH 4.5 (19). 125I-LH (170,000 cpm) mixed with 100 μg of LH is submitted to electrophoresis for 1 hour 45 min at 8 mA per tube. At the end of the electrophoresis, the gels are either stained with 1% Amido schwarz in 7% acetic acid solution, or frozen at -20° on Dry Ice, cut at 1-mm thickness, dissolved as described by Ward et al. (15) and counted in a liquid scintillation counter. A tracing of the stained gel is shown below the fractionation. Identical distribution of radioactivity is obtained with and without staining. B, quantitative precipitin curve comparing the antigenicity of LH and 125I-LH (40,000 cpm per tube). The protein precipitate is assayed by the method of Lowry et al. (14). The molecular weight of LH is assumed to be 30,000. C, binding and elution of 125I-LH (5.5 × 10^6 cpm) dissolved in 0.9 M NaCl, 0.01 M potassium phosphate, pH 7.3, applied on a column of anti-LH IgG Sepharose (1 × 0.4 cm) equilibrated with the same buffer. Fractions (0.15 ml) are collected (10).](http://www.jbc.org/)

![Table I. Comparison of biological activity of LH and 131I-LH when assayed by ovarian ascorbic acid depletion test (15).](http://www.jbc.org/)
Binding Assay—The assay for specific binding of \( ^{125}I \)-LH to membranes is a slight modification of that used to measure specific binding of insulin to intact fat cells (20, 21) and fat cell membranes (22).

Briefly, membranes are incubated at 23°C to equilibrium in 0.2 ml of Krebs-Ringer bicarbonate buffer containing 1% albumin (w/v) and \( ^{125}I \)-LH (\( 10^{-11} \) to \( 10^{-8} \) M). Three milliliters of ice-cold Krebs-Ringer bicarbonate buffer containing 1% albumin (w/v) are added, and the contents are passed through cellulose acetate EGWP filters positioned with vacuum. The filters are washed under vacuum with 3 times 3 ml of ice-cold Krebs-Ringer bicarbonate buffer containing 1% albumin (w/v). Every determination of binding is performed in triplicate, and for every such determination parallel, triplicate samples are performed in the presence of native LH (100 \( \mu \)g per ml) to determine the correction for nonspecific binding of LH. As it has been stressed by others (22), it is imperative that such corrections be performed in order to determine “specific” LH binding accurately. Nonspecific binding to EGWP filters in the absence of membranes is 0.3% of the input. Plasma membranes which have been heated to 90°C for 5 min do not bind more than 0.5% of the input. The filters are dissolved in 15 ml of Bray's scintillation fluid containing 4% Cab-O-Sil and counted in a liquid scintillation counter as described. Counting efficiency is 60%.

Enzyme Effect—Specific procedures used for digestion of plasma membranes with enzymes are described in the appropriate tables. Generally, the membranes are incubated with the enzyme in Krebs-Ringer bicarbonate buffer containing 1 to 0.1% albumin (w/v) for 10 to 20 min at 37°C, then washed twice by centrifugation using the same buffer (18,000 x g for 10 min). The pellet is resuspended in Krebs-Ringer bicarbonate buffer containing 1% albumin (w/v), and binding of \( ^{125}I \)-LH is then performed.

RESULTS

Binding as Function of \( ^{125}I \)-LH Concentration—The specific binding of \( ^{125}I \)-LH to purified plasma membranes obtained from bovine corpus luteum is a saturable process with respect to \( ^{125}I \)-LH concentration (Fig. 3). Specific binding of \( ^{125}I \)-LH can be detected at a concentration as low as 3 \( \mu \)g per ml (\( 1 \times 10^{-10} \) M), and saturation is obtained at 300 \( \mu \)g per ml (\( 1 \times 10^{-8} \) M).

Displacement of Bound \( ^{125}I \)-LH by Native LH and Other Gonadotropins—\( ^{125}I \)-LH specifically bound to plasma membranes is displaced by increasing concentrations of native LH in a fashion predicted by the near-identity of these 2 molecules (Fig. 4). Other polypeptidic hormones which possess LH activity such as HCG and PMSG are able to compete for the receptor site of LH. However, their affinity is about 10 times less than that of LH.

Of the two gonadotropins, HCG is the most potent (Fig. 5). Other polypeptidic hormones which possess LH activity such as HCG and PMSG are able to compete for the receptor site of LH. However, their affinity is about 10 times less than that of LH. The affinity of human LH or HCG using lactoperoxidase as a catalytic agent are not satisfactory for ovine LH. With their affinity of human LH or HCG using lactoperoxidase as a catalytic agent are not satisfactory for ovine LH. With their aspergillus luteinalg process (17). The cell concentration is \( 1 \times 10^{6} \) cells per ml. Incubation is performed in the presence of LH, \( ^{125}I \)-LH, and without either.

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Hormones which are free of LH activity such as FSH (Fig. 6) compete very poorly for the LH binding site. Its affinity all-
FIG. 3. Specific binding of $^{125}$I-LH to bovine corpus luteum plasma membranes as a function of the concentration of LH. Plasma membranes (100 μg of protein) is incubated at 23° for 20 min, in 0.2 ml of Krebs-Ringer bicarbonate buffer, 1% albumin (w/v), and various concentrations of $^{125}$I-LH. Specific binding and correction for nonspecific adsorption of LH is determined as described in the text.

FIG. 4. Effect of varying concentrations of LH on the binding of $^{125}$I-LH to plasma membranes of bovine corpus luteum. Suspensions containing 150 μg of plasma membrane in 0.2 ml of Krebs-Ringer bicarbonate buffer, 1% albumin (w/v) are incubated with $1 \times 10^{-9}$ M $^{125}$I-LH and the indicated amount of LH for 20 min at 23°. Specific LH binding is determined as described in the text and corrected for nonspecific binding.

Comparison to the early portion of the curve of Fig. 3 (10$^{-20}$ to 10$^{-9}$ M). It is impossible to achieve high enough concentrations of plasma membrane to see a plateau of binding since at high membrane concentration the filter clogs.

Binding as Function of Time—The specific binding of $^{125}$I-LH to plasma membrane of corpus luteum at 23° reaches a maximum at 12 min and then stays constant. In contrast, nonspecific binding reaches a maximum at 4 min (Fig. 7B).

Effect of Temperature on Rate Constant of $^{125}$I-LH Receptor Interaction—The rate of binding of $^{125}$I-LH to its receptor increases with increasing temperature. It reaches a maximum at 37° (Fig. 8). The rate constants of association can be calculated from these data since they obey second order kinetics. At 23° the rate of association $k_1$ is $2.17 \times 10^6$ M$^{-1}$ s$^{-1}$ while at 10° it is $4.2 \times 10^6$ M$^{-1}$ s$^{-1}$. The rate of dissociation decreases with decreasing temperature (Fig. 8). The dissociation data obey first order kinetics, and the half-life of the complex is 5 min at 23° and 16 min at 10°. At 23° the dissociation constant is $2.4 \times 10^{-3}$ s$^{-1}$, while at 10° it is $7.18 \times 10^{-4}$ s$^{-1}$. Values of the equilibrium constant indicate similar binding of LH to its receptor at low temperatures as compared to high temperatures (Table II).

The dissociation constant has also been computed from equilibrium data. With increasing concentration of $^{125}$I-LH the hormone binding to plasma membrane follows the equation $Y = S_{LM}/U + 1/K_{LM}$ where $S_{LM}$ is the number of LH binding sites, $U$ is the unbound LH, and $K_{LM}$ is the equilibrium constant of the LH-receptor interaction. $K_{LM}$ is measured as $3.4 \times 10^6$ M$^{-1}$ and $S_{LM}$ 474 fmoles per mg of membrane protein (Fig. 9A). Binding data can also be plotted in the form of a Scatchard
Fig. 7. A, specific binding of $^{125}$I-LH to plasma membranes of bovine corpus luteum as a function of the concentration of plasma membranes in the medium. The incubation medium contains the various amounts of plasma membranes in 0.2 ml of Krebs-Ringer bicarbonate buffer, 1% albumin (w/v), and $1 \times 10^{-9}$ M LH. After incubation for 20 min at 23°C, the specific binding (-----) and nonspecific binding (O---O) of LH is determined as described in the text.

B, time dependence of specific (-----) and nonspecific (O---O) LH binding to plasma membranes of bovine corpus luteum. The incubation medium containing 150 pg of plasma membrane in 0.2 ml of Krebs-Ringer bicarbonate buffer, 1% albumin (w/v), and $1 \times 10^{-9}$ M $^{125}$I-LH. Specific and nonspecific binding is determined at various times as described in the text.

plot (Fig. 9B). This requires the assumption that the steady-state in binding is achieved after 15 min at 23°C. From the slope of such a plot an apparent dissociation constant of $3 \times 10^{-9}$ M is obtained which agrees well with the dissociation constants computed by double inverse plot or computed directly from the rate of association and dissociation of the $^{125}$I-LH-receptor complex.

Table II

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Association rate ($k_a$)</th>
<th>Dissociation rate ($k_d$)</th>
<th>Dissociation constant from $k_a/k_d$</th>
<th>Dissociation constant from equilibrium $d_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>$2.17 \times 10^{-9}$</td>
<td>$2.46 \times 10^{-9}$</td>
<td>$1.13 \times 10^{-9}$</td>
<td>$3 \times 10^{-9}$</td>
</tr>
<tr>
<td>10°C</td>
<td>$4.2 \times 10^{-9}$</td>
<td>$7.18 \times 10^{-9}$</td>
<td>$1.71 \times 10^{-9}$</td>
<td>$3 \times 10^{-9}$</td>
</tr>
</tbody>
</table>

Effect of Salts and pH on LH Binding—The effect of different ionic constituents of the Krebs-Ringer bicarbonate buffer on the binding of $^{125}$I-LH to plasma membrane of corpus luteum is shown in Table III. Ca$^{2+}$ has no effect on the binding of $^{125}$I-LH to its receptor between 1 and 10 mM, but concentrations higher than 20 mM reduce the binding by 20%. When NaCl is present in a concentration higher than 0.5 M, the binding capacity is considerably reduced, and at 2 M no binding of $^{125}$I-LH is observed. KCl has the same effect. The specific binding of $^{125}$I-LH to plasma membrane occurs over a relatively narrow range of pH (Fig 10). Maximum binding is observed at pH 7.4 and 7.6. At pH 6 or 8 the binding capacity is only 30% of the binding capacity observed at pH 7.6. However, the low binding capacity of plasma membrane at low pH values such as pH 5 or high pH values such as pH 9 is fully reversible since incubation of plasma membrane at those pH values followed by washing and resuspension in pH 7.6 fully restores their capacity to bind $^{125}$I-LH.

Effect of Enzymes on LH Binding—The effects of several enzyme preparations on the binding of $^{125}$I-LH to plasma membranes of corpus luteum are shown in Table IV. Purified preparations of trypsin, chymotrypsin, and pepsin do not affect it. Digestion of plasma membrane preparations with neuraminidase increases the binding capacity of the plasma membrane, while digestion with phospholipase lowers it to half the control value. Collagenase, one of the enzymes we used to dissociate luteal cells
Table III

Effect of various salts on specific binding of ¹²⁵I-LH to plasma membranes of bovine corpus luteum

Plasma membranes are suspended in small volumes of 0.025 M Tris-HCl pH 7.4. Aliquots (10 μl, containing 200 μg of protein) are added to 300 μl of the indicated buffer containing 1% albumin (w/v). Samples are incubated at 24°C for 35 min with 1 × 10⁻⁹ M ¹²⁵I-LH to determine specific binding as described in the text. The pH of all buffers is adjusted at 7.4. Results are expressed in femtomoles per mg of protein.

<table>
<thead>
<tr>
<th>Salt conditions</th>
<th>Specific binding ¹²⁵I-LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M Tris-HCl</td>
<td>100 ± 1.3</td>
</tr>
<tr>
<td>+ 1 mM CaCl₂</td>
<td>96 ± 1.4</td>
</tr>
<tr>
<td>+ 10 mM CaCl₂</td>
<td>100 ± 1.5</td>
</tr>
<tr>
<td>+ 20 mM CaCl₂</td>
<td>88 ± 1.8</td>
</tr>
<tr>
<td>0.1 M Sodium phosphate</td>
<td>100 ± 1.2</td>
</tr>
<tr>
<td>+ NaCl 0.15 M</td>
<td>104 ± 1.2</td>
</tr>
<tr>
<td>+ NaCl 1 M</td>
<td>56 ± 1.2</td>
</tr>
<tr>
<td>+ NaCl 2 M</td>
<td>74 ± 1.2</td>
</tr>
<tr>
<td>+ KCl 0.15 M</td>
<td>17 ± 1.2</td>
</tr>
<tr>
<td>+ KCl 2 M</td>
<td>20 ± 1.8</td>
</tr>
</tbody>
</table>

Fig. 10. Binding of ¹²⁵I-LH as a function of pH. Plasma membranes, 120 μg, are incubated in 0.05 M sodium phosphate buffers (pH 5.0 to 8.0), 0.1 M NaCl, 1% albumin (w/v), and 1 × 10⁻⁹ M ¹²⁵I-LH. After incubation at 24°C for 20 min, specific binding is determined as described in the text.

Table IV

Effect of phospholipases on specific binding of ¹²⁵I-LH to plasma membranes of bovine corpus luteum

Plasma membranes of corpus luteum (1 mg per ml) are incubated in Krebs-Ringer bicarbonate buffer containing 1% albumin (w/v) with the indicated enzyme in the presence and absence of CaCl₂ (10 mM) for 30 min at 37°C. The membranes are washed thoroughly and suspended in the same buffer. The binding of ¹²⁵I-LH (1 × 10⁻¹⁰ M) is then measured as described in the text. The phospholipase C used here is from Clostridium perfringens, the phospholipase A from Vipera russelli or from bee venom. Results are expressed in femtomoles per mg of protein.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific ¹²⁵I-LH binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80 ± 6.6</td>
</tr>
<tr>
<td>Phospholipase A (150 μg per ml)</td>
<td>42.3 ± 1.5</td>
</tr>
<tr>
<td>Phospholipase A (100 μg per ml)</td>
<td>41.1 ± 1.6</td>
</tr>
<tr>
<td>Phospholipase C (150 μg per ml)</td>
<td>41.8 ± 1.5</td>
</tr>
<tr>
<td>Phospholipase C (100 μg per ml)</td>
<td>41.5 ± 2.5</td>
</tr>
</tbody>
</table>

Table V

Effect of proteolytic enzymes, hyaluronidase, collagenase, and neuraminidase on binding of ¹²⁵I-LH to plasma membranes of bovine corpus luteum

One-milliliter suspensions of plasma membranes containing 1 mg of protein per ml of 0.025 M Tris, 1 mM CaCl₂ are incubated at 37°C for 30 min with various concentrations of proteolytic enzymes, collagenase, hyaluronidase, DNase, and neuraminidase. In the case of trypsin, chymotrypsin, and protease, at the end of the incubation soybean trypsin inhibitor is added in a 10-fold excess. The plasma membranes are washed with 10 ml of Krebs-Ringer bicarbonate buffer-1% albumin (w/v). Samples are incubated at 2-1°C for 35 min with ¹²⁵I-LH (1 X 10⁻⁹ M) is then measured as described in the text using 1 × 10⁻⁹ M ¹²⁵I-LH. Results are expressed in femtomoles per mg of protein.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific ¹²⁵I-LH binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>102 ± 9</td>
</tr>
<tr>
<td>Trypsin (100 μg per ml)</td>
<td>107 ± 8</td>
</tr>
<tr>
<td>Chymotrypsin (100 μg per ml)</td>
<td>98 ± 7</td>
</tr>
<tr>
<td>Pepsin (100 μg per ml)</td>
<td>102 ± 8</td>
</tr>
<tr>
<td>Protease (100 μg per ml)</td>
<td>64 ± 4</td>
</tr>
<tr>
<td>Collagenase (250 μg per ml)</td>
<td>98 ± 8</td>
</tr>
<tr>
<td>Hyaluronidase (100 μg per ml)</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>DNase (100 μg per ml)</td>
<td>112 ± 6</td>
</tr>
<tr>
<td>Neuraminidase (100 μg per ml)</td>
<td>224 ± 1.3</td>
</tr>
</tbody>
</table>

from bovine corpus luteum (16), does not affect the binding of LH. However, hyaluronidase does reduce it by 30%. Whether this effect is due to proteolytic activity present in this impure preparation or to hyaluronidase itself is not clear. Protease, an impure collection of different types of proteolytic enzymes, greatly diminishes the binding capacity of bovine corpus luteum.

Discussion

Study of the interaction of LH with its receptor site has been made possible by obtaining preparations of ¹²⁵I-LH with high specific activity (1800 Ci per mm) which exhibits normal immunological and biological activity. Our earlier attempts to study the interaction of ¹²⁵I-LH with its receptor site failed because we had labeled the hormone using the chloramine T method. Even though we have attempted to minimize the damage to the proteins caused by chloramine T by working with an equimolar ratio of chloramine T to hormone, the specific activity of the hormone (300 Ci per mm) is low, as is the incorporation of ¹²⁵I (11). To raise the specific activity of ¹²⁵I-LH, chromatography on a diethylaminoethyl cellulose is required to separate noniodinated from iodinated molecules. This purification step is time-consuming and difficult to do in view of the quantity of radioactivity manipulated. Also, even though the immunological activity of the final product is not greatly altered (except that the precipitin curve of the iodinated LH does not coincide with that of LH), its biological activity is on an average 20% lower than that of LH, and when binding studies are conducted, saturation of sites even at high concentrations of LH (10⁻⁷ to 10⁻⁶ M) is not observed. So denaturation, which is manifested in only small changes in immunological or biological activity, results in large amounts of spurious binding. Also, in the case of human LH or HCG, when chloramine T is used as an iodinating agent, the ratio of chloramine T to hormone, the length of exposure, and the temperature are critical factors for the iodination of human LH or HCG if binding studies are to be conducted with it after-
hormone by LH. Kammerman et al. (26), looking at the com-
petition by LH α and LH β has also been analyzed. It is known
that the average 1 molecule of LH contains 1 molecule of
125I-LH. The immunological and biological properties of 125I-LH
are similar to those of the native hormone and when binding
studies of 125I-LH with its receptor, present in plasma membrane
of corpus luteum, are investigated, saturability of the receptor
site is obtained.

Evidence for the specificity of the LH-cell interaction is further
strengthened by the failure of several polypeptide hormones to
compete with LH for binding to cells. We observe no displace-
ment of 125I-LH (2 × 10⁻¹⁰ M) by growth hormone, prolactin,
adrenocorticotropin, or glucagon at concentrations as high as 3
× 10⁻⁸ M. However, native LH is able to compete with 125I-LH
for binding. Two other gonadotropins which possess LH ac-
tivity, HCG and PMSG, are able to compete for the receptor site
with an activity 10 times less than that of ovine LH, hardly an
astonishing fact since we are working with a homologous system.
Ovine LH and bovine LH have been shown to be similar, and the
receptor site of plasma membrane of bovine corpus luteum should
then have a higher affinity for ovine LH than for gonadotropin of
unrelated species such as human or horse. The binding com-
petition by LH α and LH β has also been analyzed. It is known
that LH α and LH β have a low steroidogenic activity (9)
and ascorbic acid depletion activity (6, 8, 24, 25) when compared
to LH. This is reflected in their low binding affinity for the LH
receptor site present in plasma membrane fraction of bovine
corpus luteum. Their ability to compete with LH is 2% for LH
α and 1% for LH β. These values represent, respectively, a 100-
and 200-fold molar excess over LH and suggest that neither sub-
unit alone is sufficient to convey significant receptor activity.
The binding activity of LH α and LH β is comparable to the values
reported by us for their steroidogenic activity in bovine
plasma membrane (8) and similar to their activity in the radioligand
receptor assay (32). The competition of ovine FSH is negligible.
Displacement is observed only at a 10,000-fold excess of FSH over
LH and most probably reflects the residual contamination of this
hormone by LH. Kammerman et al. (26), looking at the com-
petitive binding of HCG and FSH in porcine granulosa cells,
have reported similar data.

Of special interest to us is the effect of different enzymes on
the binding of 125I-LH to its receptor in plasma membrane fraction
of bovine corpus luteum. We have recently reported the isolation
and the maintenance in tissue culture (26) of metabolically active
bovine luteal cells, obtained from corpus luteum by an enzymatic
treatment (16). Of the three enzymes that we are using for the
cellular dissociation, two, collagenase, and trypsin, do not affect
the binding site of LH. The third, hyaluronidase, reduces the
binding affinity for LH by a third. While it is not yet clear
whether this effect is due to proteolytic activity present in this
enzyme preparation or to hyaluronidase itself, it is evident
that its use for cell dissociation presents some dangers if one
wants to study the binding of LH to luteal cells. However, the
kinetics of binding 125I-LH to cell suspensions obtained from
corpus luteum shows the same characteristics as those described
for the binding of 125I-LH to plasma membrane. It is possible
that the hyaluronidase can affect many more receptors in isolated
plasma membranes than it can in intact cells when they are
associated together or in suspension. Among the other enzymes
which reduce the binding capacity of plasma membrane is
leucine, which reduces it by half. A similar effect of leci-
thinase is observed on the binding of glucagon to plasma mem-
branes of rat liver (28). In contrast, it has been reported that
the binding of insulin to fat cell membrane is increased 3-fold
after treatment by leucine (29). Among the enzymes which
increase the binding capacity of bovine plasma membrane frac-
tion is neumuninidase, which gives a 2-fold increase.

The dissociation constant for LH in bovine plasma mem-
brane calculated from equilibrium kinetics (3 × 10⁻³ M) is com-
parable to that (1 × 10⁻³ M) calculated from the ratio of the
rate constants. Both of these are similar to the values ob-
tained for the stimulation of progesterone release in bovine luteal
cells (9). The association of 125I-LH to membranes is a rapid
phenomenon which takes no more than 12 min to be completed
at 23°C. The rate of dissociation at the same temperature is also
rapid, and the half-life of the complex is 5 min at 23°C. These
results are in contrast with those of Catt et al. (30) who have studied
the binding of another gonadotropin (HCG) to a testis homoge-
nate. They find that the uptake of 125I-HCG at 24°C continued to
increase for 24 hours. The half-life of the complexes at 20°C
is also of the same order of time. The association rate constant
calculated from this date for the initial reaction velocity is 10
times lower than the association rate constant for the binding of
125I-LH to receptor site in plasma membrane of corpus luteum
(127 ± 10⁶ M⁻¹ s⁻¹ versus 2.17 × 10⁵ M⁻¹ s⁻¹). However, due
to the slow rate of dissociation of the complexes the equilibri-
um constant (6 × 10⁻¹⁰ M⁻¹) determined from the association and
dissociation rate constants is similar to the one we find (1 × 10⁵
M⁻¹). The dissociation constant of the LH-receptor complex
can also be compared to that found by Leidenberger and Reichert
for the binding of HCG to rat testicular homogenate (3 × 10⁻¹⁰
M) (10). In the case of lutoiding hormone, the specific binding
has been studied by others using Leydig tumor cells with radiol-
imunoassay as a means to detect the specific binding of LH (34)
or ovarian slices (32). In the case of the Leydig tumor cells a
binding constant of 1.5 × 10⁻⁸ M was obtained, and for the
ovarian slices a value of 3.6 × 10⁻⁸ M was given. These results
obtained by different laboratories can be greatly affected by the
systems used, the method to determine the binding of the hor-
monc to its receptor, and the way the hormone is labeled. In
this context, kinetic studies done with plasma membrane frac-
tions, where proteolytic activity ordinarily present in crude
extracts is minimized and where we can eliminate the possibility
of nonspecific binding to intracellular structure membrane and
to connective tissue structures, have a definite advantage over
these done with crude homogenate and slices of organs. Also,
the method we use permits direct and quantitative observations
of complex formation between LH and its receptors at concentra-
tions of hormone normally present in biological fluid. Direct
measurement of the dissociation equilibrium constant is possible,
based on the rate constant for association and dissociation,
rather than indirect measurement based on equilibrium condi-
tions which give apparent dissociation constants.

The interaction of LH with its receptor sites present in plasma
membrane is identical with that found with isolated bovine
luteal cells and gives us reasonable assurance that this interaction
is biologically significant and represents the initial interaction
that leads to the activation of the adenylate cyclase present in
plasma membrane. Despite the loss of biological activity when
the cell is disrupted, the specific binding of LH to its receptor site
should permit its identification during the purification of the
receptor sites which will eventually lead to its isolation.

Note Added in Proof—Since this manuscript was submitted
for publication Channing and Kammerman (33) have described
the characteristics of gonadotropin receptors of porcine granulosa
cells and Lee and Ryan (34) have reported the binding of human
LH to homogenates of luteinized rat ovaries. \( K_D \) of 7.9 \( \times 10^{-10} \) M
was found.

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