Luteinizing Hormone Receptors: Specific Binding of Human Luteinizing Hormone to Homogenates of Luteinized Rat Ovaries

(dissociation constant/number of binding sites)

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ABSTRACT Binding of human [125 I]luteinizing hormone to homogenates of luteinized rat ovaries is dependent upon time, temperature, and pH and is saturable. Injection of human chorionic gonadotropin *in vivo* or addition of unlabeled human chorionic gonadotropin or human or ovine luteinizing hormone *in vitro* inhibits binding, whereas follicle stimulating hormone or prolactin is without effect. The dissociation constant for binding of luteinizing hormone receptor is 0.79 nM. The number of binding sites is 94 femtomol/mg of wet weight. NaCl, KCl, MgSO_4 , and CaCl_2 at concentrations below 10 mM have no effect on binding, while all salts significantly inhibit binding at 150 mM. Binding of [125 I]luteinizing hormone to its receptors is destroyed by proteolytic enzymes and by phospholipase C and D. The total binding activity is quantitatively recovered in the $2000 \times g$ pellet of the homogenate.

Concentration of radioiodinated luteinizing hormone (LH) and human chorionic gonadotropin (CG) by mouse and rat ovaries *in vivo* has been observed (1-4). More recently, we and others have reported the existence of specific receptors for LH and human CG in slices of luteinized rat ovaries (5) and homogenized preparations of normal rat (6, 7) or mouse tumor Leydig cells (8). The present report deals with the binding of human LH to receptors in homogenates of luteinized rat ovaries and describes some of the properties of the interaction.

MATERIAL AND METHODS

The procedure for radioiodination of human LH was a modification of the method of Greenwood *et al.* (9). Briefly, 25 μl of human LH (25 μg) was added to 25 μl of 0.5 M phosphate buffer (pH 7.5) containing 1.0 mCi of Na^{125}I (Cambridge Nuclear, carrier-free, about 17 mCi/ μg of I). The reaction was initiated by addition of 10 μl of chloramine-T (25 mg/10 ml of 0.5 M phosphate buffer). After 1 min of reaction in an ice bath, 250 μl of sodium metabisulfite (2 mg/10 ml of 0.5 M phosphate buffer) was added, followed by 100 μl of 1% KI. [125 I]LH was purified by gel filtration on Biogel P10. The [125 I]LH obtained had a specific activity of 30-40 Ci/g of LH (about 1 atom ^{125}I /2 LH molecules) and more than 96% of the radioactivity was precipitated with 10% phosphotungstic acid.

Holtzman rats primed with pregnant mare's serum gonadotropin (PMSG) and human CG were used 6-9 days after they were given human CG (10). Luteinized ovaries were sliced

and homogenized with a Teflon-glass grinder in 10 volumes (v/w) of 40 mM Tris·HCl (pH 7.4) containing 5 mM MgSO_4 . Six strokes were performed in two 30-sec periods, while the grinder was kept in an ice bath. Homogenates were strained through four layers of gauze before use.

In the standard binding assay, 4 ng of human [125 I]LH, 5-mg wet weight of homogenate, and other test substances were incubated in a final volume of 1.0 ml in 40 mM Tris·HCl (pH 7.4) containing 5 mM MgSO_4 and 0.1% bovine-serum albumin (fraction V). At the end of 30 min of incubation at 37°, 1 ml of ice-cold Tris buffer was added to the assay medium, which was immediately filtered with suction through a Millipore filter (pore size 0.45 μm) previously washed with 4% bovine-serum albumin to reduce nonspecific binding. The adsorbed material was washed with another 10 ml of ice-cold buffer. The radioactivity on the filter was measured in a gamma spectrometer.

Purified human LH (22870-1B) was prepared in this laboratory. Another purified human LH (LER-960) was obtained from the National Pituitary Agency. Pure human CG was a gift of Dr. Robert Canfield, Columbia University. Ovine LH-S11, ovine follicle stimulating hormone (FSH)-S7, and ovine prolactin-S6 were obtained from the National Institutes of Health, Bethesda, Md. PMSG and human CG used for priming rats were purchased from Ayerst Laboratories. Na^{125}I was purchased from Cambridge Nuclear Co. Trypsin, bacterial protease, DNase, RNase, and phospholipase D were purchased from Sigma Chemical Co., and phospholipase C, RNase, and α -chymotrypsin were from Worthington.

RESULTS

Binding as a Function of Incubation Time and Temperature.

Fig. 1 shows that total binding of human [125 I]LH to ovarian homogenate is dependent on incubation time and temperature. At 37°, equilibrium was reached within 30 min. In other experiments, not shown here, there was no further change in binding during an additional 1-hr incubation. In contrast, nonspecific binding (that which occurred in the presence of an excess of unlabeled hormone) was independent of incubation time. This nonspecific component constituted only 0.65% of the total radioactivity in the incubation vessels and 3.0% of the total binding at 37° after 30 min of incubation*. Specific

Abbreviations: LH, luteinizing hormone; CG, chorionic gonadotropin; FSH, follicle stimulating hormone; PMSG, pregnant mare's serum gonadotropin.

* About 70% of this nonspecific binding represented adsorption of unbound [125 I]LH to Millipore HAWP filters previously treated with 4% bovine-serum albumin. EHWP filters bind less labeled LH.

FIG. 1. Binding of human $[^{125}\text{I}]\text{LH}$ to ovarian homogenates as a function of incubation time and temperature. The human $[^{125}\text{I}]\text{LH}$ bound is plotted as a function of incubation time at each temperature. The homogenate concentration was 5 mg/ml. The concentration of human $[^{125}\text{I}]\text{LH}$ was 9.4 ng/ml. Nonspecific binding at 37° ($\times$ — $\times$) was determined by the addition of a high concentration of human CG (200 IU/ml).

binding amounted to 21% of the radioactivity added to the flask. These results indicated that crude homogenates from ovaries primed with PMSG and human CG are useful in studies of properties of LH-luteal tissue interactions.

Effect of Homogenate Concentration. A nonlinear relationship exists between the concentration of homogenate used and the amount of labeled LH bound (Fig. 2). A maximum was approached by a homogenate concentration of 10 mg/ml. In contrast, the nonspecific binding showed a linear relationship to the concentration of homogenate.

Binding as a Function of pH of the Medium. A marked decrease in binding occurred below pH 6.0 and above pH 8.0, while maximum binding appeared to occur at about pH 7.4 (Fig. 3). Since reasonably good binding occurred at pH 6.0 with citrate-phosphate buffer, the decreased binding at low values of pH appears to be a hydrogen-ion effect rather than an effect of buffer composition.

Effects of Concentration of Various Ions on Binding of Human LH. MgSO_4 , CaCl_2 , NaCl , and KCl did not significantly effect binding of LH at concentrations below 10 mM (Table 1). At concentrations of 50 mM, KCl had no significant effect, NaCl produced moderate inhibition of binding, and CaCl_2 or MgSO_4 inhibited binding by about 50%. All salts significantly inhibited binding at concentrations of 150 mM.

TABLE 1. Effects of concentration of various ions on binding of human LH

Salt	cpm of human $[^{125}\text{I}]\text{LH}$ bound					
	0	0 + EDTA	1 mM	10 mM	50 mM	150 mM
MgSO_4	10,700	11,400	11,850	10,300	5,500	2900
CaCl_2	11,200	—	11,000	11,000	5,900	2500
NaCl	11,200	—	11,700	11,000	8,600	5100
KCl	11,200	—	10,500	11,700	10,000	5500

Four ng/ml of human $[^{125}\text{I}]\text{LH}$ and 5 mg/ml of ovarian homogenate were incubated in 40 mM Tris buffer containing the indicated concentration of various ions. Each value is the mean of two determinations. Concentration of EDTA is 1 mM.

FIG. 2. Effect of homogenate concentration on binding of human $[^{125}\text{I}]\text{LH}$. The assay medium contained 6 ng/ml of human $[^{125}\text{I}]\text{LH}$ and the indicated concentration of homogenates. Control incubations ($\times$ — $\times$) were performed in the presence of 200 IU/ml of human CG.

Displacement of Binding of Human $[^{125}\text{I}]\text{LH}$ by Various Hormones In Vivo or In Vitro. Binding was considered to be 100% in the absence of unlabeled gonadotropins. In the pres-

FIG. 3. Binding of human $[^{125}\text{I}]\text{LH}$ as a function of pH of the medium. Ovaries were homogenized in distilled water as described in *Methods*. From pH 3.1–6.0, 40 mM citrate-phosphate buffer was used. From pH 7.4–9.7, 40 mM Tris buffer was used.

FIG. 4. Displacement of binding of human $[^{125}\text{I}]\text{LH}$ by various hormones *in vitro*. Binding assays were performed as described in *Methods*, except for the addition of indicated hormones. ▲, prolactin; ■, FSH; ×, ovine LH; ○, human LH; ●, human CG.

ence of unlabeled pure human CG, pure human LH, or partially purified ovine LH, binding was progressively inhibited with increasing concentrations of protein (Fig. 4). Complete inhibition was observed in the presence of 400 ng/ml of unlabeled human CG. Ovine FSH did not inhibit binding except at high concentrations that were compatible with LH contamination. Prolactin had no effect on binding, even at a concentration of 100,000 ng/ml. *In vivo* injection of human CG also caused inhibition of binding of human $[^{125}\text{I}]\text{LH}$ to ovarian homogenate in the *in vitro* binding assay. When 50 IU of human CG per rat were injected, *in vitro* binding was inhibited to 16.2% of that with a control group injected with saline. Inhibition was virtually complete when 200 IU of human CG per rat was given (Table 2).

Binding as a Function of $[^{125}\text{I}]\text{LH}$ Concentration. Nonspecific binding that occurred in the presence of a high concentration (200 IU/ml) of human CG was related linearly to concentrations of $[^{125}\text{I}]\text{LH}$. When these nonspecific components were subtracted from total binding, a saturable binding curve was obtained (Fig. 5). The binding process followed Michaelis-Menten kinetics, and the equilibrium dissociation constant (K) and number of binding sites (n) could be obtained by a Scatchard plot (Fig. 6) from the data in Fig. 5. The relationship used was $[\text{Bound}]/[\text{Free}] = 1/K ([\text{Binding sites}] - [\text{Bound}])$. The value for K is 0.79 nM and for n , 286 pM or 94 fmol/mg wet weight tissue. Correction was made for the nonlinear relationship of weight of tissue to binding illustrated in Fig. 2.

Properties of LH Receptors. Table 3 shows that binding activity was remarkably reduced by treatment of the homog-

FIG. 5. Binding as a function of concentration of human $[^{125}\text{I}]\text{LH}$. (×—×) total binding of human $[^{125}\text{I}]\text{LH}$; (○—○) nonspecific binding obtained in the presence of 200 IU/ml of human CG; (— — —) specific binding, correction made by subtraction of nonspecific binding from total binding.

FIG. 6. Scatchard plot of data for specific binding of human $[^{125}\text{I}]\text{LH}$ illustrated in Fig. 5. The slope is equivalent to $1/K$; the X intercept equals the number of binding sites. Molecular weight of human LH was taken as 33,000.

enate with the following proteolytic enzymes: trypsin (100 and 250 $\mu\text{g}/\text{ml}$), α -chymotrypsin (100 $\mu\text{g}/\text{ml}$), and bacterial protease (100 $\mu\text{g}/\text{ml}$). Phospholipase C (250 $\mu\text{g}/\text{ml}$), phospholipase D (250 $\mu\text{g}/\text{ml}$), and RNase (100 $\mu\text{g}/\text{ml}$) also destroyed binding activity. DNase had no effect.

Effect of Centrifugal Fractionation. Ovarian tissue was homogenized and centrifuged at various speeds (Table 4), and the pellets were resuspended in the same Tris buffer. Measurement of the binding capacity of various fractions showed that most of the receptors could be recovered at low centrifugal force. At $2000 \times g$, binding protein was completely sedimented (Table 4).

DISCUSSION

The present studies, based on a homogenate preparation, indicate that luteinized rat ovaries contain highly specific

TABLE 2. Inhibition of binding of human $[^{125}\text{I}]\text{LH}$ by *in vivo* injection of human CG

	cpm of $[^{125}\text{I}]\text{LH}$ bound	%
Saline	15,900	100
Human CG, 50 IU per rat	2,600	16.2
Human CG, 200 IU per rat	100	0.6

Binding assays *in vitro* were performed as described in *Methods*, except the indicated amounts of human CG were given intravenously 1.5 hr before the rats were killed. Each group contained three rats. Each value is the mean of four determinations.

TABLE 3. *Effect of enzymes on binding of human [¹²⁵I]LH*

Enzyme	Concentration (μg/ml)	% Binding
None		100
Trypsin	100	12.9
Trypsin	250	9.2
α-Chymotrypsin	100	6.7
Bacterial protease	100	1.2
Phospholipase D*	250	74.7
Phospholipase C*	250	52.2
DNase	100	103
RNase, Sigma	100	76.3
RNase, Worthington	100	77.6

Homogenates at a concentration of 5.5 mg/ml in 40 mM Tris buffer were incubated with the enzymes at the indicated concentrations. At the end of 30 min at 37°, enzymes were removed by centrifugation followed by two washes. Each value represents the mean of three determinations.

* Digestion was performed in the presence of 10 mM CaCl₂. For phospholipase D, the pH of incubation was 5.6.

receptors for LH. The LH-receptor interaction is a saturable process and dependent on pH and temperature. With the slice preparation (5), significant binding of LH (a tissue:medium ratio greater than 1) was not seen until after about 15 min of incubation. With the homogenate preparation, significant binding occurs within 1 min, and a maximum is approached within 15–20 min. Since Dorrington (11) has indicated that LH stimulated adenylate cyclase activity of ovarian homogenates within 2 min, the demonstration of rapid binding of LH takes on functional significance.

Our earlier observation suggests that there is a close parallel between binding and biological activity of LH. The potency of various pituitary gonadotropins estimated by inhibition of binding is in good agreement with a classical bioassay such as ovarian ascorbic acid depletion (5). The subunits of LH (ovine and human) and human CG do not compete favorably with native LH for binding to the receptor (12), nor do the radioiodinated subunits of human CG bind significantly to ovarian homogenates (unpublished observation). The isolated subunits of LH and human CG have little or no biological activity (13, 14). Moudgal (8) has shown that binding of LH to Leydig cells correlates well with stimulation of testosterone production.

The dissociation constant (0.79 nM) calculated on the basis of equilibrium data from the homogenate preparation is nearly the same as that (0.71 nM) calculated from equilibrium data (unpublished observation) from the 2000 × *g* fraction of ovarian homogenate. Both of these dissociation constants compare favorably with the dissociation constant (0.72 nM) derived from the ratio of the dissociation and association rate constants (unpublished data). At low concentrations of LH, a cooperative effect was noted in the Scatchard plot (Fig. 6). The same feature was also found in estrogen-receptor preparations (15).

The anatomic location of the LH receptor can only be

TABLE 4. *Effect of centrifugal fractionation on binding of human [¹²⁵I]LH*

Fractions	cpm of [¹²⁵ I]LH bound	%
Whole homogenate	18,100	100
Pellet (700 × <i>g</i> , 15 min)	15,900	88
Pellet (1000 × <i>g</i> , 15 min)	17,200	95
Pellet (2000 × <i>g</i> , 15 min)	18,700	103
Pellet (4000 × <i>g</i> , 20 min)	19,900	110

Homogenates prepared as described in *Methods* were diluted to 25 mg/ml and centrifuged in 40 mM Tris buffer at the indicated centrifugal forces. Each value is the mean of three determinations.

partially defined. Localization of human CG and LH in ovaries by autoradiography indicates accumulation in the luteal cells (2, 3). Subcellular localization of the receptor in or on luteal cells is less certain. The centrifugation data presented herein indicated that all receptor activity was sedimented at 2000 × *g* in Tris buffer, suggesting a plasma membrane localization. However, our previous data obtained on slices incubated with labeled LH and centrifuged in 0.25 M sucrose indicate that most of the labeled LH sedimented in the 110,000 × *g* pellet (12). This discrepancy is probably related to the difference in viscosity of the two centrifugation media.

A lipoprotein nature of the LH receptor is suggested by the inactivation of binding by treatment of the homogenate with proteolytic enzymes, phospholipase C and phospholipase D. The inhibition caused by RNase is not readily explained.

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