Purification and properties of human chorionic gonadotropin/lutropin receptor from plasma-membrane and soluble fractions of bovine corpora lutea

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Plasma-membrane and soluble fractions containing human chorionic gonadotropin/lutropin receptor were prepared from bovine corpora lutea by ultracentrifugation. The plasma-membrane and soluble fractions were studied for physicochemical properties, namely pH, protein concentration, temperature, time and effects of various enzymes, salts and gangliosides. The receptor preparations obtained from the plasma-membrane and soluble fractions appeared to be identical in their properties. Each fraction was purified individually by sucrose-density-gradient centrifugation, which resulted in a partial dissociation of the hormone-binding subunit from the intact functional receptor unit, which consists of both hormone-binding (regulatory) and adenylate cyclase-associated (catalytic) subunits. The fractions containing the functional receptor unit were further purified by gel filtration on Sepharose-6B and chromatography on concanavalin A-Sepharose. The 'receptor' was finally purified by affinity chromatography on a column of controlled-pore glass covalently coupled to human chorionic gonadotropin. The purified receptor from the plasma-membrane and the soluble fractions contained binding capacities of 901 000 and 87 000 fmol of human chorionic gonadotropin/mg of protein. Yields of 0.02 and 0.22 mg of protein were obtained from 250 g of bovine corpora lutea, which represents a 10 000- and 1000-fold increase respectively in the specific binding with ^{125}I -labelled human chorionic gonadotropin. Immunization of rabbits with a partially purified receptor fraction generated antibodies that specifically inhibited the binding of the ^{125}I -labelled human chorionic gonadotropin to the receptor.

The presence of receptor molecules that specifically bind polypeptide hormones on the cell membrane have been described in the plasma membranes (Raftery *et al.*, 1971; Cuatrecasas, 1972; Dufau *et al.*, 1973, 1974a; Shiu & Friesen, 1974) as well as in the soluble fractions of the target cells (McGuffin *et al.*, 1976; Conti *et al.*, 1978; Pahnke & Leidenberger, 1978). Attempts towards the isolation of the receptor for peptide hormones from plasma membranes by affinity chromatography of detergent-solubilized plasma membranes (Cuatrecasas, 1972; Shiu & Friesen, 1974; Dufau *et al.*, 1975) have resulted in partially purified preparations of low yield and poor stability. There are no reports on the

isolation of receptors from the soluble fractions of target cells. The need for the purification of the receptors in a stable form and in sufficient quantities for biochemical characterization and for studies on the mechanism of hormone-receptor interaction has become increasingly obvious. In the present paper we describe the purification and characterization of human chorionic gonadotropin/lutropin receptors from plasma membranes as well as from soluble fraction prepared from bovine corpora lutea.

Materials and methods

Preparation of plasma-membrane and soluble fractions of bovine corpora lutea (Scheme 1)

The 'plasma-membrane' and 'soluble' fractions were prepared by minor modification of the method described previously (Haour & Saxena, 1974). All procedures were performed at 4°C. Ovaries were

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removed in the slaughterhouse from cows immediately after death, frozen in liquid N₂ and stored at -60°C until used. The plasma-membrane fraction was solubilized in the detergent Triton X-100 for further purification, as described below.

Analytical methods

Protein determination. The protein contents of the fractions obtained during purification were determined by the method of Lowry *et al.* (1951), against a bovine serum albumin standard containing the same concentration of Triton X-100. The plasma

membranes were heated at 100°C with 1 M-NaOH for 30 min before determination of protein. The turbidity produced due to Triton X-100 was removed by centrifugation at 10000 g, and the absorbance of the clear supernatant was read at 750 nm.

Preparation of ¹²⁵I-labelled human chorionic gonadotropin for binding studies. ¹²⁵I-labelled human chorionic gonadotropin was iodinated by the method of Miyachi *et al.* (1972) with the use of lactoperoxidase. Human chorionic gonadotropin, containing 12000 i.u./mg, was a gift from Dr. O. P.

* Trasylol or aprotinin (Sigma Chemical Co., St. Louis, MO, U.S.A.); 10000 kallikrein-inactivator units/ml.

Scheme 1. Preparation of plasma-membrane and soluble fractions of bovine corpora lutea

Bahl (State University at Buffalo, NY, U.S.A.) and from Dr. R. E. Canfield (College of Physicians and Surgeons, Columbia University, New York, NY, U.S.A.). The crude reaction mixture was purified by gel filtration on a 0.7 cm $\times$ 30 cm column of Sephadex G-100, pre-equilibrated with 5% (w/v) bovine serum albumin, and elution with 50 mM-sodium phosphate buffer, pH 7.2, containing 0.1% bovine serum albumin. The specific radioactivity of the labelled hormone was determined as described previously (Saxena, 1976) and was in the range of 30–35 μ Ci/ μ g.

Determination of binding of 125 I-labelled human chorionic gonadotropin. Hormone-binding assay for particulate fractions was performed as described previously (Saxena, 1976). The detergent-solubilized plasma-membrane and soluble fractions were assayed by the method of Pandian & Bahl (1977), with minor modifications. The incubation mixture contained 100 μ g of receptor protein in 100 μ l of buffer and 60 000 c.p.m. of 125 I-labelled human chorionic gonadotropin/100 μ l of buffer without and with unlabelled gonadotropin equivalent to 100 ng in 100 μ l of buffer per tube. Incubation was performed at 4°C for 16 h. The incubation was terminated by successive additions of 0.5 ml of 0.2% bovine γ -globulin in phosphate-buffered saline (50 mM-sodium phosphate buffer, pH 7.0, containing 0.9% NaCl and 0.1% NaN₃) and 1.5 ml of 20% (v/v) polyethylene glycol in phosphate-buffered saline at 4°C. The contents of the tube were mixed and left to stand at 4°C for 10 min and centrifuged at 2000 g for 15 min. The precipitate was resuspended in 1 ml of Tris/HCl buffer (10 mM-Tris/HCl buffer, pH 7.2, containing 1 mM-MgCl₂ and 1 mM-CaCl₂) containing 0.1% bovine serum albumin and 0.1% (v/v) Triton X-100 and mixed with 1 ml of 20% (v/v) polyethylene glycol. After centrifugation, the amount of radioactivity in the pellet representing bound 125 I-labelled gonadotropin was determined. The specific binding and number of binding sites were calculated by the procedure of Scatchard (1949).

Enzyme determinations. The 5'-nucleotidase (EC 3.1.3.5) and ATPase (EC 3.6.1.3) activities were measured by the methods of Widnell & Unkeles (1968) and of Muñoz *et al.* (1968) respectively. The absorbances were measured at 820 nm against water as the blank (Chen *et al.*, 1956). The cytochrome c oxidase (EC 1.9.3.1) activity was determined by following the rate of oxidation of the dithionite-reduced cytochrome c at room temperature in a 1 cm cuvette in a Beckman spectrophotometer at 550 nm (Cooperstein & Lazarow, 1951). The adenylate cyclase (EC 4.6.1.1) and glucose 6-phosphatase (EC 3.1.3.9) activities were determined respectively by the method of Salomon *et al.* (1974) and of Aronson & Touster (1974) as described by Salomon *et al.* (1977). Both cyclic AMP and cyclic

GMP were determined by using radioimmunoassay kits purchased from New England Nuclear (Boston, MA, U.S.A.) in accordance with the procedure of Steiner *et al.* (1972).

Characterization of the plasma-membrane, detergent-solubilized plasma-membrane and soluble fractions

The binding ability of the plasma-membrane fraction, detergent-solubilized plasma membranes and the soluble fractions to 125 I-labelled human chorionic gonadotropin were compared under various conditions. In all experiments samples (400 μ g of protein) of each fraction in 100 μ l of Tris/HCl buffer and 60 000 c.p.m. of 125 I-labelled human chorionic gonadotropin were incubated for 1 h at 37°C in a total volume of 300 μ l. The specific binding was determined by a parallel incubation with excess (100 ng) of human chorionic gonadotropin in 100 μ l of Tris/HCl buffer per tube as described above (Saxena, 1976).

Stability. The stability of the three receptor fractions was measured during storage at 4°C, –20°C and –60°C (in liquid N₂) and in the freeze-dried state. The effect on the activity after the removal of the Triton X-100 from the detergent-solubilized receptor was examined during storage. A partially purified receptor preparation (fraction B, Fig. 1) was covalently coupled to controlled-pore glass (Post *et al.*, 1980) and its stability was compared with that of the uncoupled receptor.

Effect of receptor protein concentration. Increasing amounts of the three receptor fractions were incubated with a constant amount of 125 I-labelled human chorionic gonadotropin at 37°C for 60 min.

Effect of temperature and time. Samples of the three receptor fractions were incubated at different temperatures, namely 4°C, 22°C, 37°C, 45°C, 55°C and 65°C, for 10 min before the incubation with 125 I-labelled human chorionic gonadotropin for durations of 15 min, 30 min, 1 h, 12 h and 24 h at 4°C, 22°C and 37°C.

Effect of pH. The three receptor fractions were incubated with 125 I-labelled human chorionic gonadotropin at pH 5.5 and 6.5 in 0.1 M-sodium acetate buffer, at pH 7.0, 7.5 and 8.0 in 10 mM-Tris/HCl buffer and at pH 9.0 and 10 in 0.1 M-sodium bicarbonate buffer.

Effect of 125 I-labelled and of unlabelled human chorionic gonadotropin. Increasing amounts of 125 I-labelled human chorionic gonadotropin were added to a constant amount of the receptor fractions and incubated with a constant excess of unlabelled gonadotropin (100 ng). Alternatively, increasing amounts of unlabelled human chorionic gonadotropin were added to a constant amount of receptor and labelled gonadotropin (60 000 c.p.m.) and incu-

bated at 37°C for 1 h to calculate the capacity of binding.

Effect of protein-modifying reagents. The receptor fractions were incubated with various protein-modifying agents (Table 1) for 30 min at 37°C. The plasma-membrane fraction was centrifuged and washed with buffer after incubation. After treatment with protein-modifying reagents, the soluble receptor preparations were precipitated with 20% polyethylene glycol and centrifuged, and the supernatant was aspirated. The pellets were resuspended in 200 μ l of Triton X-100 in Tris/HCl buffer before the

addition of the labelled hormone for the determination of binding.

Effect of enzymic digestions. The receptor fractions were incubated for 20 min at 37°C with various enzymes indicated in Table 2. The plasma-membrane fraction was washed three times with 0.5 ml of Tris/HCl buffer to remove the enzymes and then incubated with labelled human chorionic gonadotropin. In the case of the soluble fraction, the 'receptor' was preincubated with 20% polyethylene glycol, and the pellets were resuspended in 200 μ l of 0.1% Triton X-100 in Tris/HCl buffer before

Scheme 2. Purification of the plasma-membrane fraction of bovine corpora lutea

incubation with ^{125}I -labelled human chorionic gonadotropin.

Effect of salts. The receptor fractions and labelled human chorionic gonadotropin were incubated in 10mM-Tris/HCl buffer, pH 7.2, containing various concentrations of CaCl_2 , MgCl_2 and KCl at 37°C for 60 min (Table 3).

Effect of gangliosides on binding of ^{125}I -labelled human chorionic gonadotropin to the plasma membranes

Ganglioside fractions were prepared from the brain tissues of chicken (Rathnam, 1966), rabbit, cat, pig, sheep and mink (the last being a gift from

Fig. 1. Sucrose-density-gradient ultracentrifugation of detergent-solubilized plasma-membrane fraction of bovine corpora lutea

For full experimental details see the text. The centrifugation was performed at $100000g$ for 8 h in a type 65 fixed-angle rotor in a Beckman ultracentrifuge. Eleven fractions (each 1 ml) were collected from each tube. (a) Samples of these fractions were analysed for binding to ^{125}I -labelled human chorionic gonadotropin (●—●), binding to ^{125}I -labelled lutropin (▼—▼), the concentration of Triton X-100 (●—●) and the concentration of protein (■—■). (b) Samples were also analysed for 5'-nucleotidase (○—○), ATPase (△—△), cytochrome c oxidase (◇—◇) and glucose 6-phosphatase (▽—▽) activities. (c) Samples were also analysed for cyclic AMP (■—■) and cyclic GMP (▲—▲) contents and adenylyl cyclase activity (□—□). On the basis of these analyses, fraction A (fractions 4 and 5) and fraction B (fractions 6–10) were pooled.

Dr. O. W. Garrigan, Seton Hall University, South Orange, NJ, U.S.A.). The plasma-membrane fraction was incubated with 25 μ g and 50 μ g of the gangliosides and 125 I-labelled gonadotropin. Specific binding with 125 I-labelled gonadotropin was also determined after preincubation of the receptor with 25 μ g and 50 μ g portions of gangliosides for 1 h at 22°C. The plasma-membrane fraction and 125 I-labelled human chorionic gonadotropin were incubated for 16 h at 4°C in a total volume of 0.3 ml as the control. The plasma membranes were separated by centrifugation at 1100 g for 30 min in a refrigerated Sorvall RC-2B centrifuge and the radioactivity of bound 125 I-labelled gonadotropin was counted.

Production of antibody to the human chorionic gonadotropin/lutropin receptor

A partially purified plasma-membrane fraction (fraction B, Fig. 1) was used as immunogen. A sample (30 mg of protein) in 10 ml of 10 mM-Tris/HCl buffer, pH 7.2, was emulsified with 10 ml of complete Freund's adjuvant (Difco, Detroit, MI, U.S.A.). Adult albino female rabbits were injected each week intradermally at 10–15 sites with 1 ml

containing 1 mg of protein for 10 weeks. The animals were then injected biweekly in a similar manner for an additional 10 weeks. The animals were bled at 4-week intervals via the ear vein. The effect of the sera on the binding of 125 I-labelled human chorionic gonadotropin to the receptor was examined for the presence of antibodies to the receptor. Normal rabbit serum was used as control.

Purification of the plasma-membrane fraction (Scheme 2)

Sucrose-density-gradient fractionation. The plasma-membrane fraction, solubilized with Triton X-100, was fractionated by continuous sucrose density gradients of 1.018–1.094, prepared in eight 13.5 ml-capacity cellulose nitrate tubes by the use of a gradient mixer (Büchler Instruments, Fort Lee, NJ, U.S.A.). The sucrose solutions were prepared in 1 mM-Tris/HCl buffer, pH 7.2, containing 0.5% (v/v) Triton X-100. Samples of the detergent-solubilized fraction (120 mg of protein in 1.2 ml) were layered on top of each of the eight gradient tubes. Eleven fractions of volume 1 ml each were collected from each tube by the aid of a syringe, and corresponding fractions from subsequent steps were analysed for

Fig. 2. Gel filtration on Sepharose-6B of detergent-soluble plasma-membrane receptor of bovine corpora lutea. A sample (100 mg of protein) of plasma-membrane fraction B (fractions 6–16) from sucrose-density-gradient ultracentrifugation was subjected to gel filtration on a 0.9 cm $\times$ 30 cm column of Sepharose-6B. The eluent was 10 mM-Tris/HCl buffer, pH 7.2, containing 0.1% Triton X-100; the flow rate was 8 ml/h, and 3 ml/fraction was collected. Samples of the fractions were analysed for specific binding with 125 I-labelled human chorionic gonadotropin (●—●), cyclic AMP content (■—■), protein concentration (■—■) and adenylate cyclase activity (□—□). On the basis of these analyses, fractions 12–22 were pooled.

protein, specific binding to ^{125}I -labelled human chorionic gonadotropin, enzyme activities, as well as for cyclic AMP and cyclic GMP concentrations as described above under 'Analytical methods'. The sucrose concentration of each fraction was determined by the measurement of the refractive index (Fig. 1). The Triton X-100 concentration of each fraction was measured by the method of Garewal (1973). The linearity of the sucrose gradient was determined by addition of 1 ml of 1% (w/v) Phenol Red to the sucrose solution of lower concentration. After centrifugation and collection of fractions, the absorbance at 400 nm was measured (Siegal & Monty, 1966).

Chromatography on Sepharose-6B (Fig. 2). Fraction B (Fig. 1), showing gonadotropin-binding activity, was applied to a column of Sepharose-6B. The fractions 12–22 (Fig. 2), showing specific binding to ^{125}I -labelled human chorionic gonadotropin, were pooled and freeze-dried.

Chromatography on concanavalin A-Sepharose (Fig. 3). The active fraction from Sepharose-6B columns was applied to a column of concanavalin A-Sepharose equilibrated at room temperature with 10 mM-Tris/HCl buffer, pH 7.2. After the elution of 1 hold-up volume of the column, the lower end of the column was plugged to prevent buffer flow for 30 min to allow the binding of the 'receptor'. The fractions 22–33 (Fig. 3), showing specific binding, were pooled and concentrated to 2 ml.

Affinity chromatography on aminopropyl-controlled-pore-glass-coupled human chorionic gonadotropin. A 250 mg portion of controlled-pore glass (pore size 300 nm) containing amino groups was activated by treatment with 5 ml of 2.5% (w/v) glutaraldehyde in 10 mM-sodium phosphate buffer, pH 7.0, for 30 min (Post *et al.*, 1980). The activated controlled-pore glass was washed with 100 ml of water followed by 30 ml of 10 mM-sodium phosphate buffer, pH 7.0. Then 3 mg of human chorionic gonadotropin was added to the controlled-pore glass suspended in the sodium phosphate buffer and the mixture shaken for 16 h. The supernatant was removed. The controlled-pore-glass-coupled gonadotropin was washed three times with 10 mM-sodium phosphate buffer, pH 7.0, shaken in 0.1 M-glycine at room temperature in order to block the free amino groups, washed with the phosphate buffer, and suspended in phosphate-buffered saline, pH 7.0, for storage at 4°C. The efficiency of the coupling, estimated from the amount of uncoupled gonadotropin in the washings, was determined by radio-receptor assay (Saxena, 1976) to be 80%.

The concentrated material from the concanavalin A-Sepharose column (containing 6 mg of protein) was applied to a 2 cm column of human chorionic gonadotropin linked to the controlled-pore glass and retained on the column for 30 min at room tempera-

Fig. 3. *Chromatography of the Sepharose-6B plasma-membrane fraction on concanavalin A-Sepharose* Pooled fractions (12–22) (32 mg of protein) from Sepharose-6B gel filtration (Fig. 2) were chromatographed on a 0.9 cm $\times$ 10 cm column of concanavalin A-Sepharose. The column was eluted with 10 mM-Tris/HCl buffer, pH 7.2, containing 0.1 M-NaCl (arrow 1), followed by 10 mM-Tris/HCl buffer, pH 7.2, containing 0.2 M- α -methyl D-mannoside and 0.2 M-NaCl (arrow 2). Fractions of volume 2 ml were collected, and samples were analysed for specific binding with ^{125}I -labelled human chorionic gonadotropin (●—●) and the concentration of protein (■—■).

ture. The column was then eluted with 10 ml of 10 mM-Tris/HCl buffer, pH 7.2, followed by 10 mM-Tris/HCl buffer, pH 5.0. The pH of the latter eluate was immediately adjusted to 7.2 with 10 mM-NaOH. The fractions were stored at 4°C and assayed for specific binding with ^{125}I -labelled human chorionic gonadotropin.

Purification of the soluble fraction (Scheme 3)

The soluble fraction was concentrated, centrifuged, dialysed and purified by sucrose-density-gradient centrifugation (Fig. 4), gel filtration on Sepharose-6B (Fig. 5) and affinity chromatography on concanavalin A-Sepharose (Fig. 6) under similar conditions to those described for the plasma-membrane fraction.

Results and discussion

Preparation of the plasma-membrane and soluble fractions from bovine corpora lutea (Scheme 1)

Bovine corpora lutea are a rich source of human

Scheme 3. *Purification of the soluble fraction of bovine corpora lutea*

chorionic gonadotropin/lutropin receptor and are readily available in large quantities from the slaughterhouse. This is an important factor in the isolation of the receptor, owing to the relatively low content of the receptor in the target organ (Davies *et al.*, 1978). From 94 g of protein present in the bovine corpora-lutea homogenate, 8.6 g and 10.2 g of protein were recovered in the plasma-membrane and soluble fractions respectively (Scheme 1), representing a recovery of only 26.8% of the total protein present in the homogenate. The specific activities of the plasma-membrane and soluble fractions increased 1.6- and 1.2-fold over that of the homogenate, accompanied by a 9- and 11-fold purification of the protein. However, as much as 72.6% of the receptor activity was lost during the preparation of the plasma-membrane and soluble fractions from the crude corpora-lutea homogenate. Owing to the stringent stereospecific requirement of the hormone-receptor binding, the loss of the binding capacity was most probably due to the denaturation of the receptor. It was observed that fresh corpora lutea, thawed even partially and restored in the frozen state, lost the hormone-binding ability completely within a few days. At -60°C , the corpora lutea were stable for several months. The

plasma membranes, however, were stable in a frozen state for up to 3 months at -20°C , for 6 months at -60°C and for up to 1 year in the freeze-dried state.

Characterization of the plasma-membrane, detergent-solubilized plasma-membrane and soluble fractions

All the three receptor preparations bound maximum ^{125}I -labelled human chorionic gonadotropin at pH 7.0, which is similar to the pH 7.2 obtained with plasma membranes (Haour & Saxena, 1974; Dufau *et al.*, 1974b). Samples of the three preparations containing 300–400 μg of protein were saturated with 60 000–80 000 c.p.m., equivalent to approx. 1.2–1.5 ng of ^{125}I -labelled gonadotropin of specific radioactivity 35 $\mu\text{Ci}/\mu\text{g}$. The effect of the time on the binding of the ^{125}I -labelled human chorionic gonadotropin to the receptor at various temperatures showed that most of the binding was obtained within 1–2 h at 37°C and at 22°C , whereas at 4°C the total binding was higher but required more than 24 h to attain saturation. The binding was decreased at temperatures higher than 37°C and was lost at 45°C , indicating the degradation of both the receptor and of the iodinated hormone.

The effect of various protein-modifying reagents is

Fig. 4. Sucrose-density-gradient ultracentrifugation of the soluble fraction from bovine corpora lutea. The clear supernatant was layered over linear sucrose density gradients (5–25%, w/v) prepared in 10 mM-Tris/HCl buffer, pH 7.2, containing 0.1% Triton X-100. After centrifugation at 100 000 g for 8 h at 4 °C, 1 ml fractions were collected. (a) Samples were analysed for 5'-nucleotidase (○—○), ATPase (△—△), cytochrome c oxidase (◇—◇) and glucose 6-phosphatase (▽—▽) activities. (b) Samples were also analysed for specific binding with ^{125}I -labelled human chorionic gonadotropin (●—●), adenylate cyclase activity (□—□) and the concentration of protein (■—■). On the basis of these analyses, the fractions 1–4 and 5–9 were pooled and designated as pooled fractions A and B respectively.

Table 1. Effects of various protein-modifying reagents on binding of ^{125}I -labelled human chorionic gonadotropin by plasma-membrane and soluble fractions of bovine corpora lutea. For full experimental details see the text. Results are given as means $\pm$ S.E.M. for four determinations.

Reagent	Specific binding of ^{125}I -labelled gonadotropin (%)		
	Plasma-membrane fraction		Soluble fraction
	Untreated	Detergent-solubilized	
Untreated control	100	100	100
Urea (2 mM)*	76 $\pm$ 2.0	75 $\pm$ 3.1	52 $\pm$ 4.2
Acetic anhydride (10 mM)*	25 $\pm$ 3.0	20 $\pm$ 2.4	23 $\pm$ 5.1
Tetranitromethane (12 mM)*	20 $\pm$ 2.5	18 $\pm$ 1.2	19 $\pm$ 2.5
N-Ethylmaleimide (10 mM)*	104 $\pm$ 2.6	100 $\pm$ 2.3	105 $\pm$ 3.1
Mercaptoethanol (10 mM)†	105 $\pm$ 2.7	105 $\pm$ 1.4	111 $\pm$ 2.1
Iodoacetamide (10 mM)†	102 $\pm$ 2.4	91 $\pm$ 2.1	112 $\pm$ 6.2

* Added in 10 mM-Tris/HCl buffer, pH 7.2.
† Added in ethanol (final concn. 5%).

summarized in Table 1 and was similar on all the three preparations. In 2 M-urea there was a 25% loss of binding in the plasma-membrane and detergent-solubilized plasma-membrane fractions, where-

as almost 50% of the activity of the soluble fraction was lost. This may be due either to the dissociation of non-covalently linked moieties in the receptor molecule or to a partial dissociation of the ^{125}I -

Fig. 5. Gel filtration on Sepharose-6B fraction B from sucrose-density-gradient ultracentrifugation of the soluble fraction from bovine corpora lutea

Pooled fraction B (100 mg of protein) from sucrose-density-gradient centrifugation of the soluble fraction (Fig. 4) was subjected to gel filtration on a 0.9 cm $\times$ 30 cm column of Sepharose-6B. The column was eluted with 10 mM-Tris/HCl buffer, pH 7.2, containing 0.1% (v/v) Triton X-100. The column was maintained at 4°C. A flow rate of 8 ml/h was obtained, and 3 ml fractions were collected. The specific binding to 125 I-labelled human chorionic gonadotropin (●—●) and the protein content (■—■) were measured in samples of the fractions. On the basis of these measurements, fractions 12–22 were pooled.

labelled gonadotropin, used in the assay, into its subunits. Acetic anhydride and tetranitromethane, which modify several amino acid residues, destroyed 75–80% of the hormone-binding activity of all the three preparations. However, after treatment with *N*-ethylmaleimide, mercaptoethanol and iodoacetamide the hormone-binding activity of the receptor was retained. Complete reduction of detergent-solubilized rat Leydig-cell membranes, however, had resulted in the loss of binding (Catt & Dufau, 1978), indicating that some of the disulphide bonds may be essential.

The loss of hormone-binding activity of all the three preparations after treatment with proteinases (Table 2) and the retention of binding in the simultaneous presence of trypsin and trypsin inhibitor indicates the essential nature of the protein moiety of the receptors. The loss of activity after treatment with trypsinogen and chymotrypsinogen may be due to the contamination with trypsin and/or chymotrypsin. The retention of the binding activity

Fig. 6. Chromatography on concanavalin A-Sepharose of pooled fractions 12–22 from Sepharose-6B purification of the soluble fraction from bovine corpora lutea

Pooled fractions 12–22 (32 mg of protein) from Sepharose-6B gel filtration (Fig. 5) were chromatographed on a 0.9 cm $\times$ 10 cm column of concanavalin A-Sepharose. Conditions of chromatography were the same as described for the plasma-membrane fraction (Fig. 3). Samples of the fractions collected were analysed for specific binding with 125 I-labelled human chorionic gonadotropin (●—●) and the concentration of protein (■—■).

after the treatment with nucleotidases suggested a lack of a role of nucleic acids in the receptor structure. Lipase and the phospholipases A and C destroyed the binding activity of the receptor preparation, indicating an essential role of lipid component, e.g. linkages of the type A, B and C present in the phosphatidylcholine component of the receptor lipid moiety (White *et al.*, 1978; p. 612). It is noteworthy that treatment of plasma membranes at 37°C for 1 h with phospholipase C (enzyme/substrate ratio 1:10, w/w) resulted in a significant increase in the binding activity, whereas higher concentrations of the enzyme resulted in a decrease (Haour & Saxena, 1974), suggesting that at lower concentrations of the enzymes some of the receptor molecules may be occluded from the lipid bilayer, whereas higher concentrations of the enzymes may destroy the essential phospholipids and thus the conformation of the receptor molecules. On the other hand, phospholipase D, specific for the cleavage of linkage D, did not affect the binding activity. Treatment with neuraminidase for only 20 min at 37°C at pH 7.2 caused up to 20–50% enhancement in the receptor activity of all the preparations. It is known that desialylation of human chorionic gonadotropin can cause a 2-fold

Table 2. *Effect of enzyme treatments on binding of ^{125}I -labelled human chorionic gonadotropin by plasma membrane and soluble fractions of bovine corpora lutea*For full experimental details see the text. Results are given as means $\pm$ S.E.M. for four determinations.

Enzyme treatment*	Specific binding of ^{125}I -labelled gonadotropin (%)		
	Plasma-membrane fraction		Soluble fraction
	Untreated	Detergent-solubilized	
Untreated control	100	100	100
Proteinases			
Trypsin	1 $\pm$ 0.2	0.7 $\pm$ 0.4	6.5 $\pm$ 2.1
Trypsin + inhibitor	99 $\pm$ 4.0	95.0 $\pm$ 2.5	101.0 $\pm$ 3.1
Trypsinogen	21 $\pm$ 3.0	2.0 $\pm$ 0.3	20.0 $\pm$ 2.2
α -Chymotrypsin	2 $\pm$ 1.2	3.0 $\pm$ 1.3	3.0 $\pm$ 1.2
Chymotrypsinogen	2 $\pm$ 0.4	1.0 $\pm$ 0.4	2.0 $\pm$ 0.2
Pronase	0	0	0
Nucleotidases			
Ribonuclease	95 $\pm$ 2.4	95.0 $\pm$ 3.4	105.0 $\pm$ 6.4
Deoxyribonuclease	91 $\pm$ 5.1	88.0 $\pm$ 2.1	100.0 $\pm$ 2.1
Lipases	0	0	0
Phospholipase A	15 $\pm$ 2.4	12.0 $\pm$ 2.1	20.0 $\pm$ 2.4
Phospholipase C	21 $\pm$ 3.1	20.0 $\pm$ 2.4	35.0 $\pm$ 3.1
Phospholipase D	96 $\pm$ 2.1	96.0 $\pm$ 4.1	84.0 $\pm$ 4.1
Glycosidases			
Neuraminidase	120 $\pm$ 3.2	158.0 $\pm$ 2.2	149.0 $\pm$ 6.1

* Added in 10 mM-Tris/HCl buffer, pH 7.2; enzyme/protein ratio 1:5, w/w.

Table 3. *Effects of various salts on binding of ^{125}I -labelled human chorionic gonadotropin by plasma membrane (A), by detergent-solubilized plasma membrane (B) and by the soluble fraction (C)*For full experimental details see the text. Results are given as means $\pm$ S.E.M. for four determinations.

Salt	Concn. of salt*	Specific binding of ^{125}I -labelled human chorionic gonadotropin (%)					
		0	0.1 mM	1 mM	10 mM	100 mM	1 M
MgSO ₄							
A	100	100	99 $\pm$ 1.2	105 $\pm$ 1.4	91 $\pm$ 2.1	46 $\pm$ 2.3	5 $\pm$ 2.4
B	100	100	93 $\pm$ 2.0	108 $\pm$ 2.1	89 $\pm$ 2.7	51 $\pm$ 3.1	12 $\pm$ 3.4
C	100	100	104 $\pm$ 3.0	122 $\pm$ 2.4	108 $\pm$ 2.9	68 $\pm$ 3.2	18 $\pm$ 3.2
CaCl ₂							
A	100	100	99 $\pm$ 0.4	114 $\pm$ 2.1	90 $\pm$ 0.2	42 $\pm$ 2.1	10 $\pm$ 2.2
B	100	100	99 $\pm$ 3.2	116 $\pm$ 3.2	94 $\pm$ 1.5	42 $\pm$ 2.7	10 $\pm$ 2.9
C	100	100	104 $\pm$ 1.4	123 $\pm$ 2.4	98 $\pm$ 2.5	46 $\pm$ 2.8	19 $\pm$ 3.0
NaCl							
A	100	100	99 $\pm$ 1.2	100 $\pm$ 2.1	100 $\pm$ 3.4	54 $\pm$ 1.4	19 $\pm$ 1.4
B	100	100	101 $\pm$ 3.1	105 $\pm$ 3.2	102 $\pm$ 2.1	60 $\pm$ 0.6	12 $\pm$ 2.1
C	100	100	100 $\pm$ 2.1	113 $\pm$ 2.1	98 $\pm$ 3.2	56 $\pm$ 1.8	16 $\pm$ 3.2
KCl							
A	100	100	104 $\pm$ 4.1	99 $\pm$ 4.1	101 $\pm$ 1.2	87 $\pm$ 2.1	24 $\pm$ 3.4
B	100	100	102 $\pm$ 2.2	94 $\pm$ 3.2	105 $\pm$ 1.4	79 $\pm$ 3.2	22 $\pm$ 1.4
C	100	100	100 $\pm$ 2.3	113 $\pm$ 2.1	91 $\pm$ 1.9	51 $\pm$ 2.1	22 $\pm$ 3.6

* 400 μg equivalent of each receptor fraction was incubated with the various salt concentrations at 37°C for 60 min in 0.1 M-Tris/HCl buffer, containing 1 mM-MgCl₂.

increase in the binding of the gonadotropin to the receptor *in vitro* (Bahl & Moyle, 1978). Whether or not the neuraminic acid plays a role in the recognition of the receptor before hormone binding

is not clear from these observations. The treatment of all the three receptor preparations with various salts showed a decrease in the activity at high concentrations (Table 3), and was similar to that

found previously with bovine plasma membranes (Haour & Saxena, 1974).

Effect of gangliosides on binding of the ^{125}I -labelled human chorionic gonadotropin to the receptor

The effect of 25 μg and 50 μg of gangliosides prepared from chicken brains on binding of ^{125}I -labelled human chorionic gonadotropin to the plasma membrane is shown in Fig. 7. Under these conditions the plasma-membrane fraction showed 50–100% increase in the binding with ^{125}I -labelled gonadotropin, especially when the gangliosides were preincubated with the membranes. These observations, though preliminary, suggest that gangliosides may play an important role in the binding of the hormone to the plasma-membrane receptor. It is noteworthy that the effect of the gangliosides was dose-related. Gangliosides from other sources such as pig, sheep, cat and mink showed results similar to that of the chicken, suggesting that the source of gangliosides need not be species-specific. Gangliosides have been identified to have special roles in cell

Fig. 7. Effect of gangliosides from chicken brain on the binding of the receptor in the plasma-membrane fraction from bovine corpora lutea to ^{125}I -labelled human chorionic gonadotropin

See the text for full experimental details. All the incubations were performed at 4°C for 16 h. (a) Percentage specific binding of control (without gangliosides) set to 100%; (b) receptor and ^{125}I -labelled human chorionic gonadotropin were preincubated for 15 min before the addition of membranes; (c) gangliosides and plasma membranes were preincubated before the addition of ^{125}I -labelled human chorionic gonadotropin; (d) receptor, ^{125}I -labelled human chorionic gonadotropin and gangliosides were incubated together without preincubation; (e) gangliosides and plasma membranes were incubated together, and gangliosides were removed by centrifugation before the addition of ^{125}I -labelled human chorionic gonadotropin; (f) ^{125}I -labelled human chorionic gonadotropin and gangliosides were preincubated before the addition of membranes. The results are shown as means $\pm$ S.E.M. for three determinations.

plasma-membrane receptors for thyroprotein (White *et al.*, 1978, p. 1219), for viruses and for pharmacologically active substances (White *et al.*, 1978, p. 616).

Antibody to the receptor

The percentage binding of the plasma-membrane fraction to ^{125}I -labelled human chorionic gonadotropin that is obtained in the presence of the antisera is shown in Fig. 8. The inhibition of the binding of the ^{125}I -labelled gonadotropin to the plasma membrane was obvious even with the first batch of serum. Second and third batches of antisera, collected 4 and 8 weeks later, showed a similar effect at dilutions of 1:1000 and 1:4000, indicating increases titres. These observations confirm that antibodies to the receptor could be generated; however, further characterization of the antibody is required to demonstrate that they were directed specifically to the receptor molecule and not to other related

Fig. 8. Inhibition by antisera to the human chorionic gonadotropin/lutropin receptor of the specific binding between the plasma-membrane receptor from bovine corpora lutea to ^{125}I -labelled human chorionic gonadotropin

The antisera from two rabbits were assayed at dilutions of 1:100 (●—●), 1:1000 (□—□) and 1:4000 (○—○). The tubes containing the plasma-membrane receptor protein (400 μg), labelled human chorionic gonadotropin (60 000 c.p.m.) and the sera at various dilutions in 10 mM-Tris/HCl buffer, pH 7.2, were incubated for 1 h at 37°C . The specific binding was determined by incubation with excess of human chorionic gonadotropin (100 ng per tube) in parallel.

protein(s) in the vicinity of the receptor. Antibodies to other receptors such as those of prolactin have been reported (Shiu & Friesen, 1976).

Purification of plasma-membrane fraction (Table 4)

Solubilization of the plasma-membrane fraction with Triton X-100 resulted in a 2-fold increase in the specific activity (201 fmol/mg) and in the removal of approx. 60% of the protein. A 50% decrease in the total binding capacity was primarily due to the insufficient concentration of Triton X-100 to solubilize all the plasma-membrane proteins (Scheme 2). The formation of large-molecular-weight micelles at higher concentrations of the detergent could also lead to the denaturation of the receptor and/or hormone molecules and interfere in the correct measurement of the binding capacity of the receptor.

During sucrose-density-gradient centrifugation, 0.5% Triton X-100 was used to prevent the precipitation of the solubilized receptor. Most of the Triton X-100 and inactive proteins were eliminated in fractions 1–3 (Fig. 1a). Two major ^{125}I -labelled-gonadotropin-binding fractions, associated with 5'-nucleotidase activity, a marker for plasma membranes (Neville, 1960), were recovered in sucrose concentrations of 15–20%. Fraction A, in tubes 4 and 5, was of lower specific activity as compared with fraction B in tubes 6–10 (Fig. 1b). Fraction A, however, did not contain any adenylate cyclase activity (Fig. 1c), whereas fraction B contained both hormone-binding activity and adenylate cyclase activity. Fraction A therefore may represent binding sites (regulatory subunit) partially dissociated from the adenylate cyclase-associated fully functional receptor unit in fraction B. Bramley & Ryan (1978), with superovulated-rat ovarian homogenates, Gospodarowicz (1973), with bovine plasma membranes, and Dufau *et al.* (1977), with Lubrol-

solubilized rat corpora lutea and Leydig cells, have also demonstrated two components of the gonadotropin receptor and proposed that the lower-molecular-weight component without adenylate cyclase activity represents the hormone-binding site or the regulatory subunit, whereas the high-molecular-weight component associated with adenylate cyclase activity represents the catalytic subunit. These observations are consistent with the existence of a hormone-binding site (the regulatory subunit) that is structurally separate and independent of the catalytic subunit associated with the adenylate cyclase (Singer & Nicholson, 1972). The subunits associate via intermediary molecules, e.g. guanylyl nucleotides (Rodbell *et al.*, 1975), to form the 'functional receptor' unit.

As shown in Table 4, fraction B showed a further removal of 75% protein, a 3-fold increase in the specific activity and approx. 70% recovery of the total binding activity. Fraction A contained the remainder of the binding activity. A greater stability of the receptor was observed during and after this step (Table 4). Hence the major loss of activity was only in the initial stage of fractionation of the corpora-lutea homogenate.

The purification of sucrose-density-gradient fraction B on Sepharose 6B is shown in Fig. 2. A major inactive protein fraction with little hormone-binding ability, cyclic AMP content and adenylate cyclase activity was eluted in the unretarded fraction. The retarded fraction, however, contained most of the binding to ^{125}I -labelled gonadotropin, cyclic AMP, adenylate cyclase activity and 7.4% of the total protein applied to the column. The specific activity increased 10-fold, i.e. to 6020 fmol/mg (Table 4).

On further fractionation on concanavalin A-Sepharose, a major protein fraction with low ^{125}I -labelled-gonadotropin-binding activity was eluted with the buffer containing 0.1 M-NaCl (Fig. 3).

Table 4. Yield and specific binding of ^{125}I -labelled human chorionic gonadotropin of fractions obtained during purification of the plasma-membrane fraction from bovine corpora lutea

For full experimental details see the text. Results are given as means $\pm$ S.E.M. of three determinations. A preparation of human chorionic gonadotropin containing 12000 i.u./mg was used as ligand.

Fraction	Protein (mg)	Binding capacity			Purification of specific binding (fold)
		Specific (fmol/mg)	$10^{-5} \times$ Total (fmol)	Recovery (%)	
Homogenate	9400.00 $\pm$ 20.0	87	8.10		1.0
Plasma-membrane fraction	860.00 $\pm$ 4.0	139	1.20	100.0	1.5
Triton X-100-solubilized	290.00 $\pm$ 2.0	201	0.58	48.3	2.7
Sucrose-density-gradient fraction B	70.00 $\pm$ 1.2	602	0.42	35.0*	6.9
Sepharose-6B fraction	4.50 $\pm$ 1.0	6020	0.27	22.5	69.2
Concanavalin A-Sepharose fraction	0.16 $\pm$ 0.3	143 900	0.23	19.2	1654.0
Controlled-pore-glass-gonadotropin chromatography fraction	0.02 $\pm$ 0.6	901 494	0.18	15.0	10362.0

* The recovery at this step does not include fraction A.

Tris/HCl buffer containing 0.2M- α -methyl D-mannoside and 0.2M-NaCl eluted a fraction containing high specific binding. The specific binding capacity increased 1650-fold, i.e. to 144 000 fmol/mg.

The concanavalin A-Sepharose-purified receptor was finally subjected to affinity chromatography on human chorionic gonadotropin coupled covalently to controlled-pore glass. The fractions eluted at pH 5 showed a 6-fold increase in the specific activity, i.e. from 144 000 to 901 000 fmol/mg, at this final step of purification.

The purified receptor represented a 10 000-fold increase in the specific activity over the crude corpora-lutea homogenate. A 15% recovery of the total activity of the plasma-membrane fraction was obtained after purification. The loss of binding capacity was due both to the actual loss of receptor in side fractions, mostly during the preparation of membranes, and to inactivation during solubilization with Triton X-100. Some inactivation of the binding capacity by various reagents during experimental procedures may not be ruled out. The purified receptor was more stable when stored in a solution at 4°C as compared with storage in a frozen or a freeze-dried state. The binding activity of the receptor, coupled covalently to aminopropyl controlled-pore glass, was stable for more than a month.

Purification of the soluble fraction (Scheme 3)

The yield and activity of the various fractions are summarized in Table 5. The soluble fraction contained approx. 11% of the total protein and 12% of the total binding activity present in the crude homogenate. The specific binding of the soluble fraction and of the homogenate was almost the same. As shown in Fig. 4, sucrose-density-gradient centrifugation of the soluble fraction resulted in the separation of a fraction B (tubes 5–9) containing 75% of the total binding capacity, with a 3-fold increase in the specific activity. Fraction B contained 5'-nucleotidase, adenylate cyclase, glucose 6-phos-

phatase and ATPase activities, but was devoid of cytochrome *c* oxidase activity, indicating that the soluble receptor fraction contained the functional receptor and was also of the plasma-membrane origin. The enzyme profile of fraction B of the soluble component (Fig. 4) being similar to the one obtained from the plasma-membrane fraction B (Fig. 1) further indicates the similarity of the two receptor preparations. The glucose 6-phosphatase activity found in fraction B may be due either to microsomal contamination or to the presence of endoplasmic reticulum, which is morphologically continuous with the plasma-membrane proteins (Emmelot & Benedetti, 1967). The presence of ATPase activity in fraction B may be accounted for by the absorption of serum soluble cytoplasmic proteins and enzymes such as ATP pyrophosphohydrolase (Lansing *et al.*, 1967). Fraction A, containing 75% of the protein, exhibited low binding to ¹²⁵I-labelled gonadotropin and low enzymic activities. It is to be noted that only one hormone-binding component was present in the presence or in the absence of detergent at this step.

As shown in Fig. 5, gel filtration of fraction B on Sepharose 6B resulted in the removal of a major unretarded protein fraction with low hormone-binding activity. Most of the receptor activity was recovered in the retarded fraction, which showed a 10-fold increase in the specific binding activity and an almost 59% recovery of the activity applied. In terms of the crude homogenate, after sucrose-density-gradient centrifugation and Sepharose 6B chromatography there was a 37-fold increase in the specific activity. Of the total activity 19% was present in the purified fraction from the Sepharose 6B column.

Further purification by chromatography on concanavalin A-Sepharose (Fig. 6) yielded a fraction, eluted by 0.2M- α -methyl mannoside in buffer containing 0.2M-NaCl, containing 0.22 mg of protein and a specific activity of 87 000 fmol/mg, indicating a 1000-fold increase in the specific activity of the crude homogenate (Table 5). The specific activity of

Table 5. Yield and specific binding to ¹²⁵I-labelled human chorionic gonadotropin of fractions obtained during purification of the soluble fraction from bovine corpora lutea

For full experimental details see the text. Results are given as means $\pm$ S.E.M. of three determinations. A preparation of human chorionic gonadotropin containing 12 000 i.u./mg was used as ligand.

Fraction	Protein (mg)	Binding capacity			Purification of specific binding (fold)
		Specific (fmol/mg)	10 ⁻⁵ $\times$ Total (fmol)	Recovery (%)	
Homogenate	9400.00 $\pm$ 20.00	87	8.1		1.0
Soluble fraction	1020.00 $\pm$ 10.20	102	1.0	100.0	1.1
Sucrose-density-gradient fraction B	255.00 $\pm$ 2.00	292	0.74	74.1	3.3
Sepharose-6B fraction	10.20 $\pm$ 1.00	3226	0.33	33.0	37.0
Concanavalin A-Sepharose fraction	0.22 $\pm$ 0.09	86 920	0.19	19.0	999.0

the purified receptor from the soluble fraction was lower, since, owing to the paucity of the material, the final step of affinity chromatography on controlled-pore glass was not performed with the soluble receptor fraction.

Of interest was the observation that the yield of the receptor in terms of protein and specific activity in the plasma-membrane fraction, namely 8.6 g and 139 fmol/mg, were comparable with the 10.2 g and 102 fmol/mg recovered in the soluble fraction. Pahnke & Leidenberger (1978) also found a water-soluble gonadotropin-binding component in rat Leydig-cell membranes. Conti *et al.* (1978) reported up to 10% recovery of the total gonadotropin/lutropin-binding capacity of the luteinizing rat ovary in the spontaneously soluble fraction. Dufau *et al.* (1977) have encountered water-soluble high-affinity follitropin receptors representing up to 20% of the total follitropin-binding capacity of rat testis Leydig cells.

In conclusion, the similarities in the properties of the receptors from the soluble fraction and from the plasma membrane (Tables 1–3), as well as the association of plasma-membrane and soluble hormone-binding fractions with 5'-nucleotidase activity, suggest that they are identical. The soluble fraction therefore may be (a) derived from the plasma membranes by partial solubilization during homogenization and purification procedures, (b) derived from 'internalized' receptors from the cytoplasm (Braendle *et al.*, 1973; Czago, 1978) or (c) derived from precursors of receptors synthesized in the cell.

The studies reported in the present paper also indicate that during purification the intact functional receptor unit can be partially separated into a hormone-binding (regulatory) and an adenylate cyclase-associated (catalytic) subunit. The purification of the human chorionic gonadotropin/lutropin receptor from the bovine corpora lutea described in the present paper has yielded a receptor fraction from the plasma membranes containing a binding capacity of 901 000 fmol of gonadotropin/mg. Even though the yields are low, availability of 500–600 g of bovine corpora lutea for processing at one time has allowed the recovery of the receptor in reasonable yields for further physicochemical and biochemical studies.

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