Biochemical Pharmacology, Vol. 40, No. 9, pp. 2093–2103, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

PURIFICATION AND CHARACTERIZATION OF THE HUMAN OVARIAN LH/hCG RECEPTOR AND COMPARISON OF THE PROPERTIES OF MAMMALIAN LH/hCG RECEPTORS*

KATHARINE ALPAUGH,† KORAKOD INDRAPICHATE, JOHN A. ABEL,† RONALD RIMERMAN and JAYANTHA WIMALASENA‡

Department of Physiology, University of Nebraska Medical Center, Omaha, NE 68105; and † Department of Biology, Lehigh University, Bethlehem, PA 18015, U.S.A.

(*Received* 28 *November* 1989; *accepted* 18 *May* 1990)

Abstract—Methods previously published by us [Wimalasena *et al.*, *J Biol Chem* **260**: 10689–10697, 1985; Wimalasena *et al.*, *J Biol Chem* **261**: 9416–9420, 1986] were utilized to solubilize the human corpus luteal leuteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor with 3-[(3-cholamidepropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and to purify the receptor by two steps of hCG-Sepharose affinity chromatography. The specific binding capacity (SBC) of the purified human receptor was 7510 pmol/mg protein, and $K_A = 2.2 \times 10^9\,M^{-1}$ when iodo hCG was competed by hCG; the yield was 4–7% of starting activity. When hLH was used in competition with hCG, specific binding capacity was 7900 pmol/mg protein and K_A $1.0 \times 10^9\,M^{-1}$. Silver staining and autoradiography demonstrated a single protein of M_r 78,000 under reducing and M_r $58–62 \times 10^3$ under nonreducing conditions. Rat ovarian LH/hCG receptor was purified by similar methods and the K_A of $3.5 \times 10^{10}\,M^{-1}$ for hCG was substantially different from the K_A for hLH which was $2.1 \times 10^9\,M^{-1}$. M_r of the rat protein was $78–82 \times 10^3$ (reduced) and $58–62 \times 10^3$ (nonreduced) when analyzed by silver staining and autoradiography. For the first time, human LH/hCG receptor has been purified to apparent homogeneity, and its M_r of 78,000 was essentially identical to the M_r values of purified rat and porcine receptors.

The initial site of interaction of luteinizing hormone (LH§) and human chorionic gonadotropin (hCG) with the ovary is believed to be a common receptor. The effects of LH on the ovary in all mammals and of hCG on the human ovary play critical roles in mammalian reproductive biology and, as such, the receptor has been studied intensively in the past decade. For detailed biochemical, immunochemical and regulation studies, the purification and characterization of the human LH/hCG R are of crucial importance. However, the low abundance and the instability of LH/hCG after solubilization from cell membrane have greatly impeded studies on the structure and function of the receptor.

We recently developed methodology for stabilization, purification and characterization of porcine ovarian LH/hCG R [1, 2]. The conclusion of our studies was that ovarian LH/hCG R is a multimeric protein composed of a single hormone binding subunit of M_r 78×10^3. Since then, several groups have reported on the purification and characterization of LH/hCG R from both the rat testis and the ovary [3–7]. However, a consensus regarding the M_r of the receptor is not evident. There is controversy about the number of putative subunits [5–9]; furthermore, the yield of purified receptor has been consistently low for the rat receptor [3, 5–7] which may indicate substantial receptor degradation during purification.

In this study, we have modified our previous methods to (a) purify and characterize the human corpus luteal receptor; (b) compare the hormone binding properties of purified rat and human receptor; and (c) compare the molecular structure of human, rat and porcine receptor. In spite of several early studies on membrane bound human LH/hCG R [10–14] and a recent report [15] on the solubilization of human LH/hCG R, the purification and the characterization of the human receptor, to our knowledge, have not been reported previously. Our work strongly suggests that the M_r values of the human, rat and porcine ovarian LH/hCG R proteins are identical; however, the hormone binding properties of the human receptor are significantly different from those of the rat and the pig.

* A preliminary account of this work was presented at the Annual Meeting of the Endocrine Society, 1988, Abstr. No. 849. Parts of the results in the manuscript were submitted by Katharine Alpaugh and Korakod Indrapichate to meet the requirements of the Ph.D. degree at Lehigh University.

‡ Address all correspondence to: Dr. J. Wimalasena, Department of Physiology, University of Nebraska Medical Center, 42nd and Dewey, Omaha, NE 68105.

§ Abbreviations: LH, luteinizing hormone; CHAPS, 3-[(3-cholamido-propyl) dimethylammonio]-1-propanesulfonate; PMSF, phenylmethylsulfonyl fluoride; oFSH, ovine follicle stimulating hormone; TSH, thyroid stimulating hormone; PRL, prolactin; hCG, human chorionic gonadotropin; CNBr, cyanogen bromide; PMSG, pregnant mare's serum gonadotropin; LH/hCG R, luteinizing hormone/human chorionic gonadotropin receptor; BSA, bovine serum albumin; βME, β-mercaptoethanol; DTT, dithiotheritol; PEG, polyethylene glycol; and SBC, specific binding capacity.

MATERIALS AND METHODS

Purification of human corpus luteal LH/hCG R

Corpora lutea irrespective of age of development (except corpus albicans) were dissected out from ovaries collected at bilateral salpingo-oophoerectomies, usually associated with radical hysterectomy. Corpora lutea were dissected from the ovarian stroma, frozen at −90°, and processed for preparation of soluble LH/hCG R as follows: The tissue (1 g) used in each study was homogenized in a glass/glass homogenizer in 5 mL of buffer, 10 mM $NaPO_4$/25% glycerol, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 μg/mL soybean trypsin inhibitor. The homogenate was processed as described before [1]. Solubilization of the membrane-bound LH/hCG R was accomplished by homogenizing the 39,000 *g* pellet (P_3) in 3-[(3-cholamide - propyl)dimethylammonio] - 1 - propanesulfonate (CHAPS) in extraction medium containing 100 μg/mL soybean trypsin inhibitor and 1 mM PMSF, and the pellet/buffer volume ratio was adjusted so that the final CHAPS concentration was 8.8 mM. Following homogenization, the extract (P_3^1) was incubated for 45 min at 4°, then diluted two times with 10 mM $NaPO_4$/25% glycerol and adjusted to bring the concentration of PMSF to 0.5 mM, soybean trypsin inhibitor to 100 μg/mL, and EDTA to 5 mM. The extract was centrifuged in an L5-65 Beckman ultracentrifuge using a Beckman Ti 70 rotor to obtain S_1. The pellet (P_5) was re-extracted as for P_3 and the soluble fraction (S_2) so generated was combined with S_1 for studies of total solubilized receptors. All steps of the procedure were performed at 4°. Solubilized LH/hCG R was routinely stored at −90°.

Affinity chromatography. The human LH/hCG R was purified by modification of previously described methods [1, 2] using highly purified hCG (CR series from the National Pituitary Agency) coupled to CNBr-activated Sepharose. Five milliliters of the solubilized receptors was mixed with 5 mL of the hCG-Sepharose gel and rocked end-to-end at room temperature for 4 hr. The slurry mixture was poured into a column and flow-through was collected as fraction 1 (see Fig. 1). The column was washed with 20 times the gel volume with buffer A (10 mM $NaPO_4$, pH 7.4, 25% glycerol, 0.1% Triton, 20 μM dioleophosphatidylcholine, 10 μM cholesterol, 0.5 M NaCl). The column was next washed with buffer A adjusted to pH 5.5 (10 times gel vol.), and the LH/hCG receptor activity was eluted with buffer A adjusted to pH 4 with 1 M acetic acid (5 times gel vol.). Fractions with the highest receptor activity were pooled and rechromatographed on a second hCG-Sepharose column as described before [1]. Fractions with receptor activity from the second affinity column were dialyzed against 10 mM $NaPO_4$, pH 7.2, concentrated by lyophilization or ultrafiltration, and aliquots were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) as described below.

Hormone binding studies. Analysis of binding of iodinated hCG to soluble LH/hCG R was performed according to previously described methods [1, 16, 17]. Equilibrium binding for Scatchard analysis was performed by displacement of bound ^{125}I-hCG by increasing concentrations of unlabeled hormones. A similar experiment was done for ^{125}I-hLH displacement with unlabeled hLH. Equilibrium binding data were analyzed with the EBDA program of G. A. McPherson, published by Elsevier. Binding to membrane-bound LH/hCG R was carried out as previously published [17]. The difference between K_A values was compared by Student's *t*-test.

Iodination of human LH/hCG R. Receptors purified through two affinity steps (1 μg) were dialyzed against 10 mM $NaPO_4$/25% glycerol (pH 7.2) at 4° for 8 hr, concentrated by partial lyophilization, and iodinated by the Iodo-bead method described before [2]. The iodinated proteins were purified through a third affinity step. Aliquots (20,000 cpm, 42% acid precipitable) from the pH 7.4 wash (unbound) fraction and from the pH 4.0 elution (bound) fraction were analyzed by SDS/PAGE under nonreducing and reducing conditions [5% β-mercaptoethanol (βME)] and autoradiographed.

Electrophoresis. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of 200 ng of LH/hCG R was carried out in slabs (7.5%) exactly according to the method of Laemmli [18] using a final concentration of 5% βME, unless otherwise specified. All electrophoresis mixtures were boiled for 3 min. Gels were processed for silver staining, using the Bio-Rad (Richmond, CA) silver staining kit as per manufacturer's instructions, or for autoradiography. Gels were exposed to Kodak X-OMAT AR film for 4–8 days at −70°. M_r standards were: myosin (205K), β-galactosidase (116K), phosphorylase *b* (95K), bovine serum albumin (68K), ovalbumin (43K), lactate dehydrogenase (36K) and carbonic anhydrase (29K).

Purification of rat luteal LH/hCG R

Immature 28-day-old rats, purchased from Charles River Laboratories, were injected with pregnant mare's serum gonadotropin (PMSG) and hCG according to standard protocols as previously described [16, 19]. In recent experiments, 17β-estradiol was injected in two doses (1.5 mg each) into the rats prior to PMSG. This regimen increased the specific activity of LH/hCG R by approximately 20% but did not change the K_A for hCG (data not shown). Ten ovaries were homogenized in 10 mL of 10 mM Tris (pH 7.4) containing 25% glycerol, 1 mM PMSF, 100 μg/mL Trypsin inhibitor. The procedure for preparation of the high speed membrane pellet and its extraction with Triton X-100 to solubilize the membrane-bound LH/hCG R was essentially as previously described [1]; briefly, the pellet was extracted in 1% Triton, 2.0 mM EDTA, 20% glycerol and 10 mM Tris (pH 7.4) for 45 min with stirring at 4°. Since a substantial fraction (approximately 20%) of total LH/hCG R of the homogenate remains bound to the pellet from the first Triton extraction, the 135,000 *g* pellet obtained following the first extraction was re-extracted with Triton X-100, and the resulting soluble fraction after ultracentrifugation was also used as a source of soluble LH/hCG R for purification.

Affinity chromatography of rat LH/hCG R on highly purified hCG-Sepharose (70% of hCG used during coupling to CNBr-Sepharose was bound) was

performed as previously described [2, 19] with the following modifications: After washing the column with 20 times the gel volume of buffer B [10 mM $NaPO_4$ (pH 7.4), 1.0 M NaCl, 0.1% Triton, 25% glycerol], the column was washed with 10 times the gel volume of buffer B adjusted to pH 5.0. LH/hCG R was eluted with buffer B adjusted to pH 4 with 1.0 M acetic acid (5x gel volume). Fractions with receptor activity were dialyzed against 10 mM Tris (pH 7.4)/25% glycerol (when activity preservation was required) or against 5 mM Tris (pH 7.4) before concentration by lyophilization for SDS/PAGE analysis.

Radioiodination of purified rat LH/hCG R. LH/hCG R (1 μg) was reacted with 0.25 mCi of iodinated Bolton–Hunter reagent [3-(4-hydroxy-phenyl)-propionic acid *N*-hydroxysuccinimide ester] (NEN-Dupont, Wilmington, DE) according to instructions supplied by the manufacturer; specific activity was estimated as 1.5 Ci/g. Similar results were obtained when iodination was carried out with Iodo-beads.

Analysis of effects of reducing agents on iodo LH/hCG R. To determine the effects of reduction, iodinated receptor was treated with increasing quantities of βME or dithiotheritol (DTT), as indicated in Fig. 4, before electrophoresis.

Other methods

Purification of porcine LH/hCG R. Porcine corpus luteal LH/hCG R was purified as described before [1]. Aliquots of 200–300 ng were analyzed by SDS/PAGE and silver staining.

Protein determination. Protein in membrane and soluble fractions from rat and human preparations was measured by the method of Bradford [20] using kits purchased from Bio-Rad Laboratories. Appropriate Triton X-100 and CHAPS concentrations were used in the blank tubes. Proteins in column fractions and purified preparations were measured using colloidal gold as described [21]. Blank tubes had the same buffer as the buffer in the protein samples. This assay is linear between 20 and 200 ng/tube of BSA used as the standard protein.

Sources

Purified hormones hCG, hLH (I-3), rLH (I-7), human prolactin, hFSH (I-3), and hTSH (RP1) were provided by the National Hormone and Pituitary Program, Baltimore, MD. The hCG used for non-specific binding (about 3000 I.U./mg) and PMSG were purchased from the Sigma Chemical Co., St. Louis, MO. Phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor, CHAPS, Triton X-100 and other reagent grade chemicals also were purchased from Sigma. CNBr-activated Sepharose 4B and Protein A-Sepharose were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Chemicals for electrophoresis were from Bio-Rad Laboratories. Aurodye™forte for protein analysis and the gel electrophoresis apparatus were purchased from Hoefer Scientific, San Francisco, CA. Carrier-free $Na^{125}I$ was purchased from the Amersham Corp., Arlington Heights, IL. Iodo-beads were purchased from the Pierce Chemical Co., Rockford, IL.

RESULTS

Solubilization and purification of human LH/hCG R

Corpora lutea were dissected and processed for measurement of LH/hCG R activity as described in Materials and Methods. The total receptor activity in the homogenate, P_1, was 2884 ± 1546 fmol/g tissue (mean ± SE, N = 16). The high-speed pellet (P_3^1) was extracted with 8 mM CHAPS (optimal concentration out of three tested) to yield P_3^1. The activity in this fraction was 1801 ± 628 fmol. Upon centrifugation of P_3^1, 805 ± 187 fmol of activity was obtained in S_1 and 474 ± 169 fmol in P_5. Re-extraction of P_5 with CHAPS yielded 330 ± 100 fmol of activity in S_2. Therefore, the total soluble activity recovered was 1135 ± 287 fmol. The binding activity in the soluble fraction was 60–80% of original activity after storage at −90° for 30 days; however, 50% of soluble activity was lost following overnight storage at 4°. The large variability in the receptor activity within the sixteen experiments above resulted from the use of corpora lutea collected at various stages of development. When corpora lutea of the same age were selected (N = 3), the SE of the activity in P_1 was 17.8% of mean activity.

Human LH/hCG R was purified by affinity chromatography according to the procedure in Materials and Methods. The column was washed with buffer A, then with the same buffer adjusted to pH 5.5, and LH/hCG R was eluted with buffer A adjusted to pH 4. Recovery at the first column step ranged between 10–16% of P_1 activity and 20–26% of S_1 activity loaded onto the column (N = 6). The active fractions were pooled and rechromatographed on a second hCG-affinity column as described previously in detail [1]. The overall yield of the highly purified receptor was 4–7% of P_1 activity and 10–14% of S_1 activity. Typical results from an affinity purification are depicted in Fig. 1. While Triton was less efficient in solubilization of the receptor than CHAPS, inclusion of Triton and the lipids in the column chromatography buffers increased overall purification yield by 15–20%. The highest activity fractions were pooled and used for further analyses as described below.

Characterization of purified human LH/hCG R

Equilibrium binding analysis of purified and soluble LH/hCG R was performed with iodinated hCG and unlabeled hCG or hLH, and the results are presented in Table 1. The K_A of the soluble receptor for hLH when measured by displacement of iodo hLH with hLH was $0.8 \pm 0.3 \times 10^9\ M^{-1}$. This value is similar to the K_A reported in Table 1 where hLH was used to displace iodinated hCG. The K_A values for hCG and hLH were not changed whether the receptors were extracted with CHAPS or Triton or if the chromatographic buffers included CHAPS/Triton as opposed to CHAPS only. These results demonstrate that the purified receptor retained the affinity of the soluble receptor for hCG and hLH and that the K_A values for hCG and hLH were similar for the purified receptors. The specificity of the receptor for hCG and hLH is demonstrated by the binding analysis in Fig. 2. Whereas both unlabeled hCG and hLH displaced bound hCG, human pro-

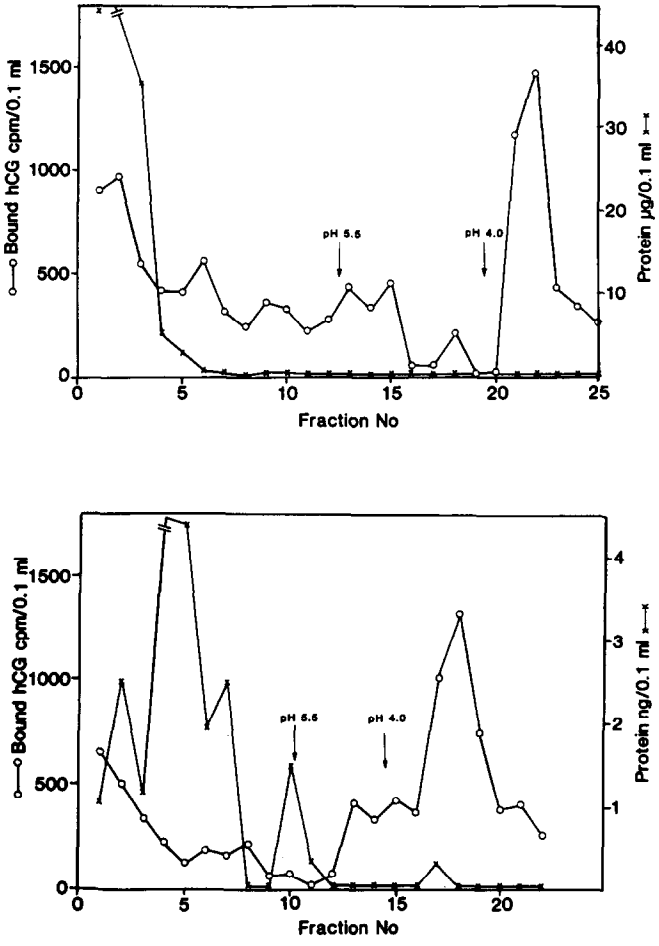


Fig. 1. Purification of the human corpus luteal LH/hCG R. Upper panel: An aliquot of soluble LH/hCG R was equilibrated with an equal volume of hCG-Sepharose gel; the mixture was poured into a glass column washed with buffer A (pH 7.4) and then with buffer A at pH 5.5. LH/hCG R activity was eluted with buffer A at pH 4.0. Lower panel: The highest activity fractions from the first affinity column were used in a second hCG-Sepharose affinity step. The highest LH/hCG R activity fractions were pooled and used for further studies as described in Materials and Methods. Note that protein is expressed as µg/mL in the upper panel and as ng/mL in the lower panel. Representative results of over twelve experiments are presented.

Table 1. Equilibrium binding parameters for human LH/hCG R

	hCG		hLH	
	SBC (pmol/mg)	K_A (M^{-1})	SBC (pmol/mg)	K_A (M^{-1})
Homogenate (P_1)	0.069 ± 0.02	6.7 ± 1.1 × 10^9	0.12 ± 0.04	1.02 ± 0.6 × 10^9
Triton extracted pellet (P_3^1)	0.13 ± 0.03	6.3 ± 0.9 × 10^{9*}	0.27 ± 0.08	0.88 ± 0.3 × 10^9
Soluble receptor (S_1)	0.21 ± 0.04	4.6 ± 0.7 × 10^9	0.46 ± 0.12	0.90 ± 0.5 × 10^9
Purified receptor	7510 ± 1291	2.2 ± 0.4 × 10^{9}†	7900 ± 1405	1.00 ± 0.3 × 10^9

Aliquots of the indicated subcellular fractions and of the hCG-affinity purified receptor were used in equilibrium binding analysis with iodo hCG and increasing concentrations of unlabeled hCG or hLH as described in Materials and Methods. Data were analyzed according to the Scatchard equation to obtain K_A and specific binding capacity (SBC). Correlation coefficients were greater than 0.94 and the data (means ± SE) are from three experiments.

* K_A for hCG of P_3^1 was significantly higher than that for the purified receptor ($P < 0.02$).

† K_A for hCG was not significantly different from that for hLH for the purified receptor ($P > 0.05$).

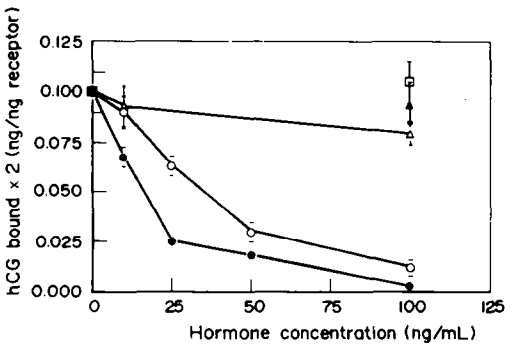


Fig. 2. Specificity of the purified human LH/hCG R. Competition binding analysis for iodo hCG and purified LH/hCG R was carried out with unlabeled hCG (●), hLH (○), hTSH (△), oFSH (▲) and human prolactin, hPRL (□) (mean ± SE, N = 3). Approximately 1 ng of iodinated hCG was used as the tracer. Where not apparent, SE bars are within symbols. Bound hCG is expressed as ng hCG bound/ng receptor protein.

lactin (hPRL), ovine FSH and hTSH were largely ineffective as competitors.

Molecular structure of the human receptor

Purified LH/hCG R was dialyzed against 2 mM $NaPO_4$ (pH 7.4), and concentrated, using a Centricell (mol. wt 30×10^3) ultrafilter (Polysciences Inc.), and approximately 200 ng/lane was analyzed by SDS/PAGE (7.5%); the results are depicted in Fig. 3 (a and b). As shown, the purified receptor was composed essentially of one protein which migrated at a M_r of 66–70 $\times 10^3$ in the presence of 0.035% βME. A minor band of variable silver staining was observed in some experiments at M_r 48 $\times 10^3$. In the absence of βME, the major protein had a M_r of 58–62 $\times 10^3$. To characterize further the M_r of the receptor protein, purified receptor was iodinated using Iodo-beads, and the iodo receptor was purified by affinity chromatography on hCG-Sepharose. The unbound wash fractions from the affinity column and the pH 4 eluate (bound or active receptor) were dialyzed and concentrated, and 20,000 cpm/lane were analyzed by denaturing SDS/PAGE ± βME (5%). As depicted in Fig. 3 (c, lane B), the iodinated LH/hCG R capable of binding to hCG had a M_r of 78×10^3. Furthermore, no other iodinated proteins were observed in the unbound wash fractions or in the iodinated receptor pool prior to the third affinity purification step (data not shown). These data further demonstrate the purity of LH/hCG R as only one iodinated receptor species was observed following iodination. Furthermore, the M_r for the human LH/hCG R obtained by silver staining under nonreducing conditions (Fig. 3a and b) was identical in M_r to the iodinated receptor observed under nonreducing conditions (Fig. 3c, lane A) and under reducing conditions as discussed below (see Fig. 5).

As a control experiment to test the specificity of the affinity purification procedure, we extracted a human liver membrane preparation in a manner identical to that of the corpus luteal preparation, chromatographed it through the two affinity steps, and analyzed the pH 4 eluted fractions by SDS/PAGE. No silver staining bands were detected (Fig. 3a), demonstrating that the liver preparation does not have proteins bound to hCG-Sepharose which could be eluted at pH 4.

Purification of the rat ovarian LH/hCG R

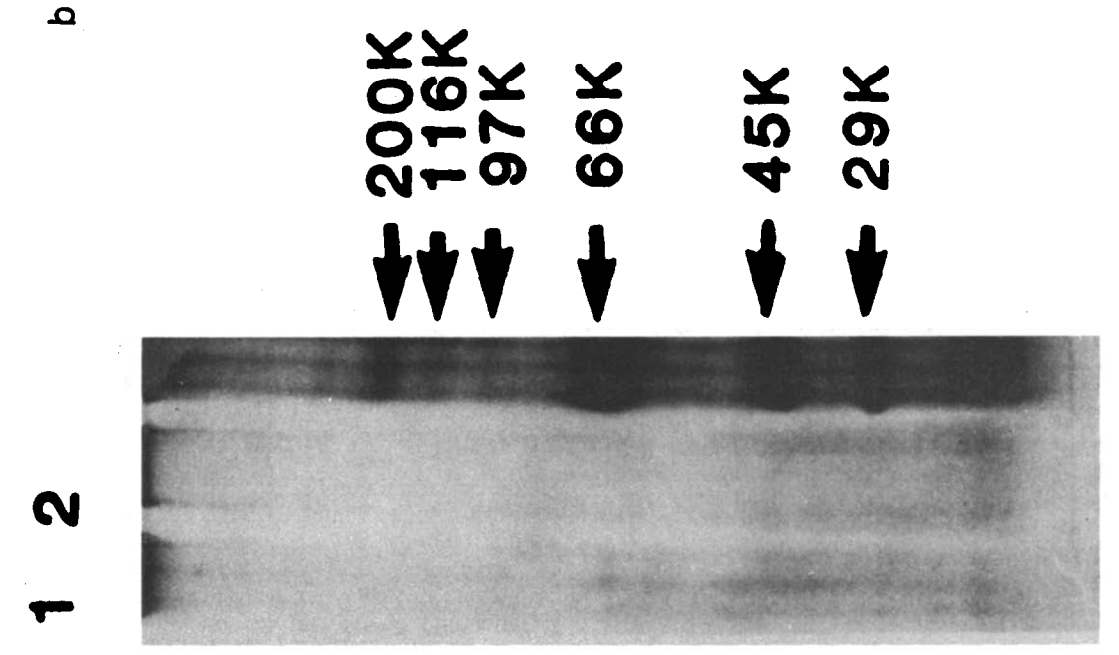
Although several papers have reported purification of the rat receptor (see Discussion), detailed binding studies for LH have not been reported. We sought to study the hormone binding properties of the purified rat receptor in some detail as the K_A values of the purified human receptor for hCG and hLH appeared to be substantially smaller in comparison to the K_A values of the purified rat receptor.

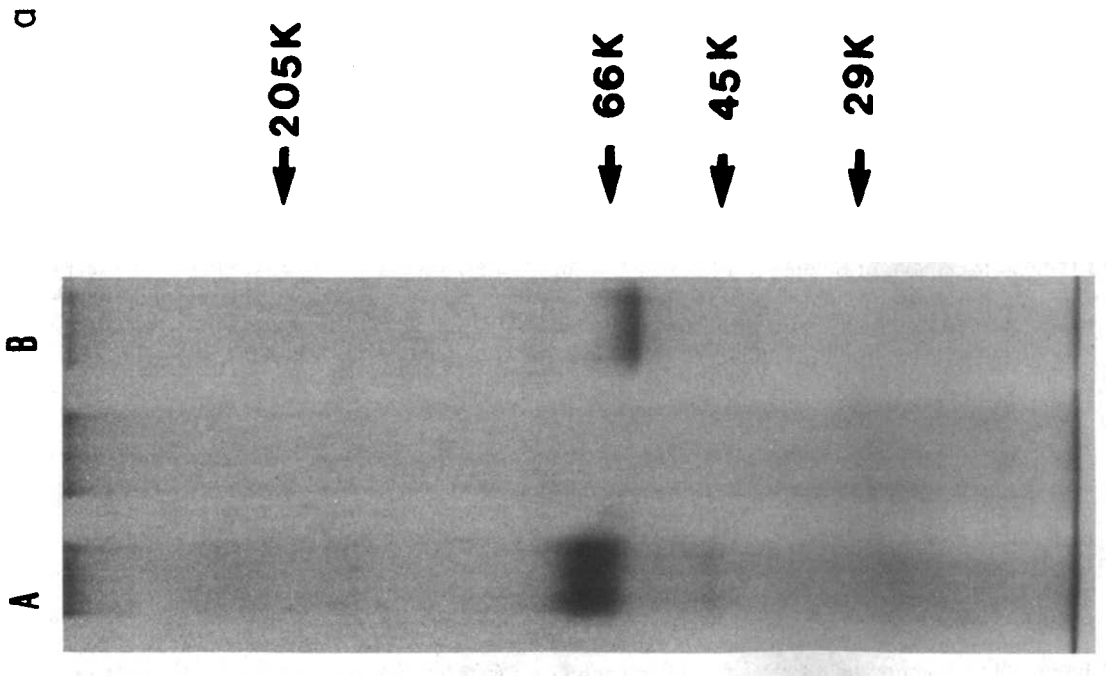
Rat receptors were purified by previously described methods [1, 2] with the additional pH 5 wash step (see Materials and Methods). Yield at the first column step was a mean of 60 ± 10% (SE, N = 6) of the activity loaded onto the column. The highest activity fractions were pooled and rechromatographed on a second affinity column with a yield of 70 ± 9% (SE, N = 6) of loaded activity. Thus, the overall average yield of activity after purification was 40% of the soluble LH/hCG R activity used in the purification or 22% of activity in the homogenate (data not shown). The specific binding capacity of the highly purified receptor was 6860 ± 90 (N = 4). We have found that purified receptor can be rechromatographed on a third affinity column with a yield of 80% (data not shown). It should be noted that chromatography of a rat liver extract through two successive hCG Sepharose columns did not produce silver staining bands in the pH 4 extract.

Characterization of purified rat LH/hCG R

The binding parameters of the purified receptor were studied by equilibrium binding methods and analyzed by Scatchard analysis. Since the rat receptor reacts *in vivo* with an LH hormone, we analyzed equilibrium binding with hLH in some detail including analysis of binding with iodo hLH prepared by published methods [22]. The binding data were as follows: when iodinated hCG was used as tracer and unlabeled hCG used for displacement, $K_A = 3.5 \pm 0.5 \times 10^{10}$; when unlabeled hLH was used as the competing hormone, $K_A = 3.1 \pm 0.7 \times 10^9$. When iodinated hLH was displaced by unlabeled hLH, $K_A = 2.1 \pm 0.3 \times 10^9$ (all of the above values are in M^{-1} and are for the highly purified receptor). For the solubilized receptor extract (S_1), $K_A = 1.6 \pm 0.8 \times 10^8\ M^{-1}$ when iodinated hCG was competed by rLH. The K_A of the purified receptor for hCG was significantly higher than that for hLH (irrespective of the tracers: iodinated hCG or hLH, at the $P < 0.01$ level). The data demonstrate that the purified rat receptor has a 15-fold higher affinity for hCG than for hLH whether hCG was displaced by hLH or iodo hLH was displaced by hLH.

The molecular structure of rat ovarian LH/hCG R was studied using SDS/PAGE following reduction and denaturation using 7.5% slab gels and silver staining. These studies demonstrated that the M_r of the receptor subunit is 78–82 $\times 10^3$ when the receptor


b
200K
116K
97K
66K
45K
29K
1 2


a
205K
66K
45K
29K
B
A

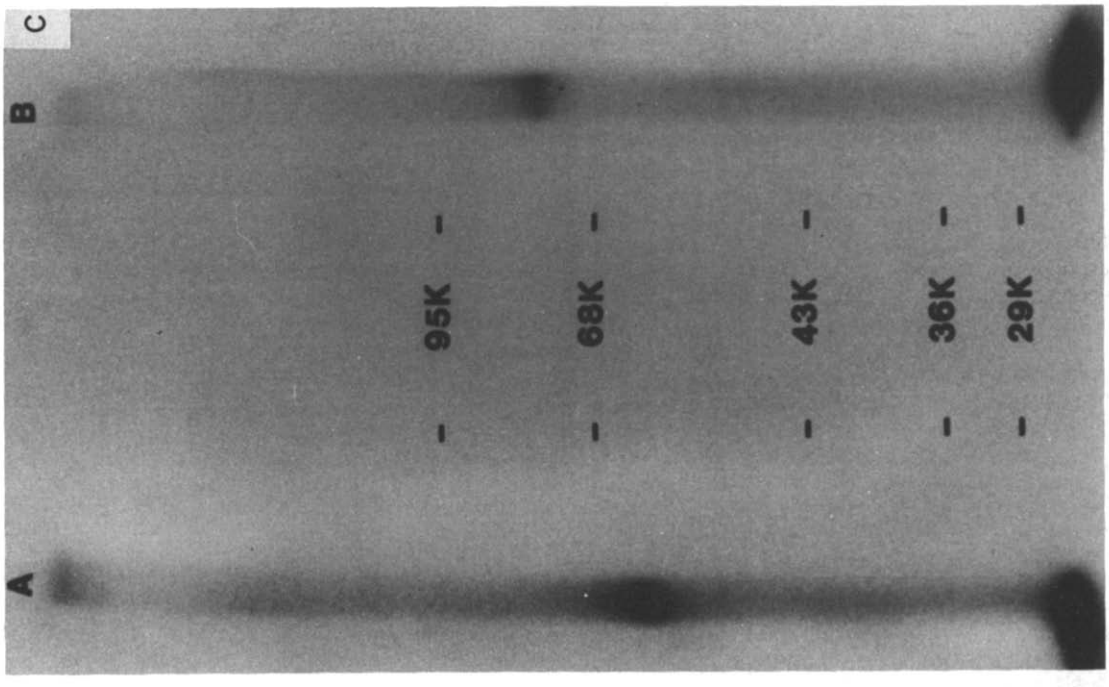


Fig. 3. (a and b) Silver stain for purified human LH/hCG R. Two hundred nanograms of purified LH/hCG R (a) was subjected to SDS/PAGE after reduction (lane A) and without reduction (B), and the gel was stained with silver. Human liver extract was passed through two affinity columns, and the pH 4 eluate from the second column was dialyzed, concentrated and analyzed on SDS/PAGE, and the gel silver stained (b, lane 1). Lane 2 (b) is a control where only protein solvent was run. Numbers indicate M_r of molecular weight markers. βME = 0.35%. (N = 3). (c) Autoradiograph of iodinated human LH/hCG R. An aliquot of iodinated LH/hCG R was purified on an hCG-Sepharose column, analyzed by SDS/PAGE, and the gel was autoradiographed. Lane A is nonreduced; lane B is reduced. (N = 2.)

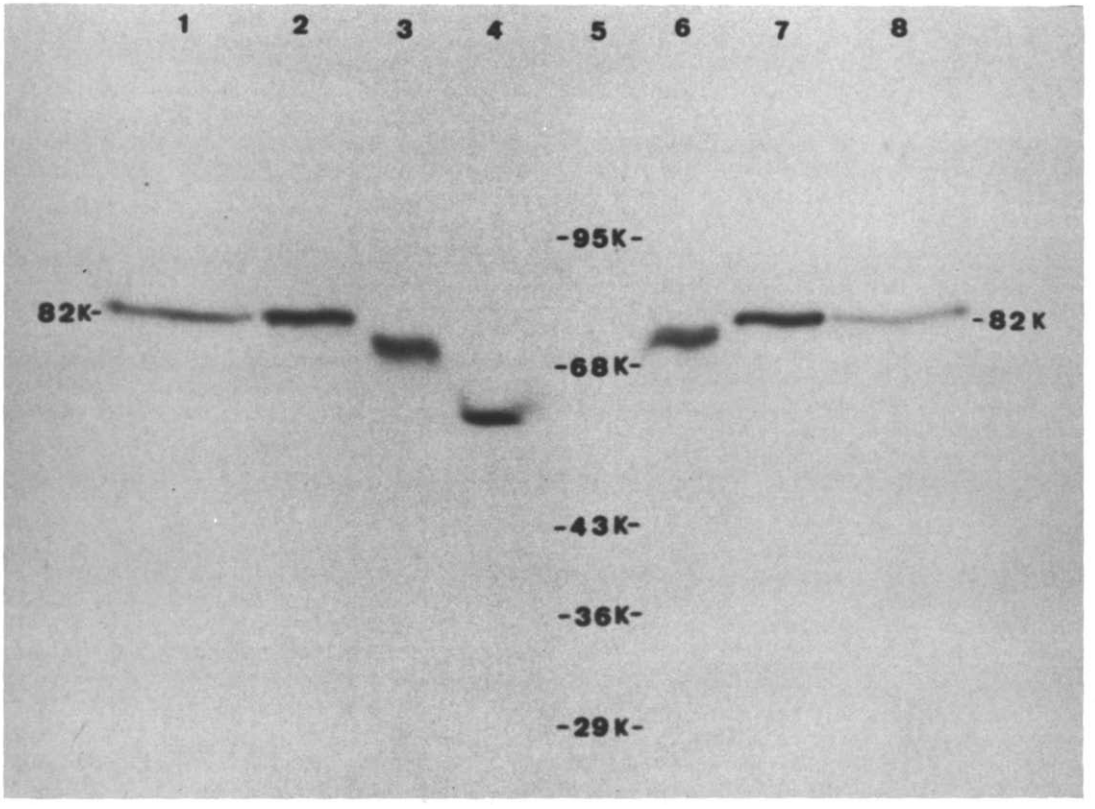


Fig. 4. Effects of reductant on iodo rat LH/hCG R. An aliquot of iodinated rat LH/hCG R was treated with no reductant (lane 4); lane 1, 5% βME; lane 2, 1% βME; lane 3, 0.1% βME; lane 6, 0.1% DTT; lane 7, 1% DTT; and lane 8, 5% DTT. Numbers are M_r markers. (N = 2.)

is reduced with 5% βME and 58–62 × 10^3 in the absence of reductant. To confirm this analysis and to study further the effects of reduction of disulfide bands, purified rat LH/hCG R was iodinated with Bolton–Hunter reagent, and its M_r was analyzed by SDS/PAGE following reduction with three concentrations of βME or DTT (Fig. 4). These studies demonstrated that M_r of the LH/hCG R depends on the concentration of reductant used during denaturation and that M_r increases from 61 × 10^3 at zero reductant to 82 × 10^3 at 5% BME or DTT. It should be pointed out that no other iodinated protein appeared in the autoradiograph.

Comparison of the structure of human, porcine and rat LH/hCG receptors

To compare the M_r values for human, porcine and rat receptors, porcine corpus luteal LH/hCG R was purified by published methodology [1] and the M_r values of human, porcine and rat LH/hCG R were compared in the same SDS/PAGE gel (T = 7.5%) (Fig. 5). These results strongly indicate that the receptor from all three species had a M_r of 76–80 × 10^3 after reduction (5% βME) and that without reduction the M_r was 58–62 × 10^3. The same analysis was repeated on SDS/PAGE at T = 6% with identical results. As demonstrated above (Figs. 3 and 4), the iodinated human receptor and the iodinated rat receptor had M_r values between 78 × 10^3 and 82 × 10^3 as expected from the silver staining data. The results in Fig. 5 also indicate that after purification of human LH/hCG R through one hCG affinity column, the major protein had a M_r of 78 × 10^3 (5% βME) after reduction and 60 × 10^3 without reduction. Since the M_r of the human receptor was 66–70 × 10^3 after reduction with 0.035% βME (Fig. 3), the M_r of the human receptor also increased with concentration of reductant used during preparation of the receptor for SDS/PAGE, as observed in Fig. 4 for the rat receptor.

DISCUSSION

The principal aim of this study was to purify the human corpus luteal LH/hCG receptor and to characterize its hormone binding properties. Additionally, a possible subunit composition of the human receptor and its hormone binding properties were compared with those of the purified rat and porcine corpus luteal receptors. Even though a number of investigators have studied the properties of crude human LH/hCG R [10–15], there are no published data on the purification or the properties of the purified human LH/hCG R.

We have purified the human LH/hCG R by the two-step hCG-Sepharose affinity chromatography procedure originally described by us [1]. The yield of the receptor activity in the pH 4 eluate of the first

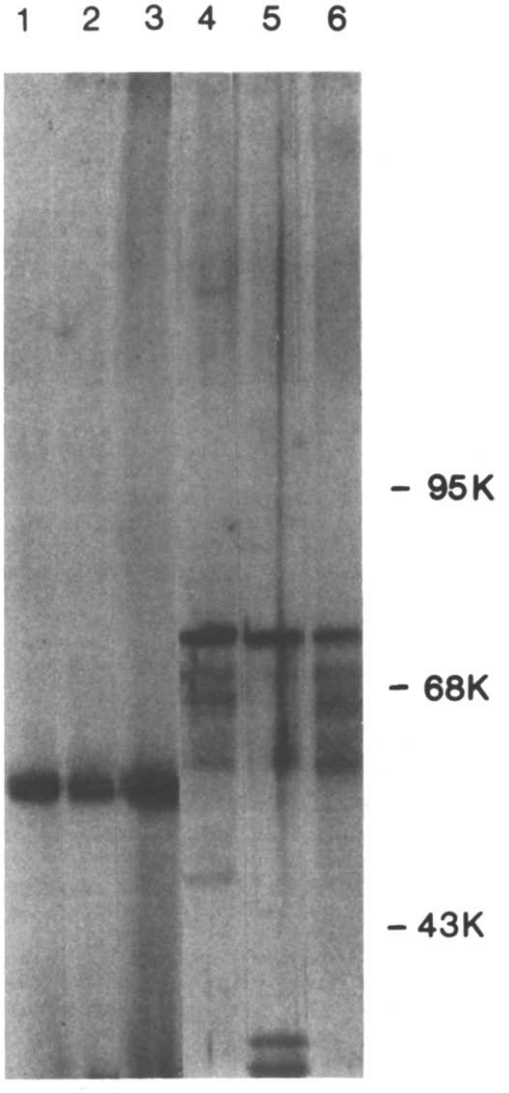


Fig. 5. Comparison of human, rat and porcine receptor M_r. Human receptor purified through one affinity step (about 200 ng) was analyzed by SDS/PAGE under reducing (lane 4) and nonreducing (lane 1) conditions. Porcine receptor (about 100 ng) purified through two affinity steps (lane 6, reduced and lane 3, nonreduced) was analyzed similarly. Highly purified rat receptor (about 100 ng) was analyzed in the same gel (lane 5, reduced and lane 2, nonreduced). The rat receptor was stored at −70° for 3 months, and low M_r degradation products were observed after prolonged storage. Note the exact identity of the R_f for the receptor bands under reducing and nonreducing conditions. (The diffuse stainings between M_r 60–70 × 10^3 are the "ghost" bands [23] which do not appear under nonreducing conditions.) (N = 3.)

affinity step was an average of 23% (range 20–26%, N = 6) and 20% of loaded activity remained unbound and was collected in the wash fractions. When the pH of the human soluble receptor preparation was lowered to pH 4 to mimic the elution conditions of the affinity column, 30% of the hCG binding activity was lost. Therefore, the major part of the loss in binding activity in the first affinity step appears to be due to acid inactivation of the receptor. After one affinity step, as shown in Fig. 5, two minor bands in addition to the M_r 78 × 10^3 protein were present in the one-affinity step purified preparation.

When the human receptor was purified through two affinity steps, a single protein of M_r 78,000 was observed by silver staining or by autoradiography of the iodinated receptor following reduction and without reduction, M_r = 58–62 × 10^3. Since the iodinated receptor bound to the hCG affinity column, the M_r 78,000 protein purified by affinity chromatography is probably the biologically active (hormone binding) human LH/hCG receptor. The autoradiographic and silver stain data strongly suggest that the human receptor has been purified to apparent homogeneity. The SBC observed for the human receptor was approximately 56% of the activity expected for a protein with a M_r of 78 × 10^3.

The SBC of the purified rat receptor reported herein and those reported on the rat receptor contrast with the achievement of theoretical specific activity for β-adrenergic receptors [24] and acetylcholine receptors [25]. The yield of the rat receptor reported herein is considerably higher than in previous reports [3–7]. The rat receptor appears to be far more stable than the human receptor as the rat soluble receptor loses 10 ± 3% of its activity on storage for 18 hr at 4°, whereas the human soluble receptor under identical conditions lost 50 ± 10% (SE) of its original activity.

A significant finding in our comparative studies on the human, rat and porcine corpus luteal receptors was that the M_r values of all three receptor proteins appeared to be very similar, if not identical, whether the receptors were reduced or nonreduced before SDS/PAGE (Fig. 5). Even though one would expect ovarian LH/hCG R from different species to be similar in molecular mass, the values reported in the literature, even for the rat LH/hCG R, are widely divergent [3, 4, 6, 7, 26–33] and the M_r values reported for the bovine ovarian LH/hCG receptor [8, 9] and porcine receptor [1, 2, 26] are different from that for the rat. The protein of M_r 78,000 ± 2,000 (SE, N = 3) that we observed under reducing conditions for all three receptors was similar in M_r to that previously reported by us for the porcine LH/hCG R [2, 19]. Under nonreducing conditions, all three receptor proteins appeared to have a M_r of 60,000 ± 2,000 (SE, N = 3). In previous studies [2], we demonstrated that the purified porcine receptor of M_r 78 × 10^3 could bind with hCG specifically; similarly, the purified iodinated human receptor of M_r 78 × 10^3 could interact with hCG-Sepharose.

Another significant similarity between human, rat and porcine receptors was the effect of reductant on the M_r of the purified receptor. Both DTT and βME produced a dose-related increase in M_r of the rat receptor; similarly, reduction increased the M_r of the human and porcine receptor. Work by other authors [6] did not show an effect of reductant on the rat receptor, whereas Sojar and Bahl [34] reported (while the present work was in preparation) results of reduction on the rat receptor that are very similar to our results. It is possible that the receptor protein has several intrachain disulfide bonds which upon reduction unfold the protein, retarding its migration

on SDS/PAGE and thereby increasing the apparent M_r of the protein.

In contrast to the similarities discussed above, the hormone binding properties of the human receptor were distinctly different from those of the rat and pig. The K_A of the purified human receptor for hCG was not significantly different from that for hLH (Table 1). In the rat system, the K_A for hCG is 10- to 20-fold higher than that for hLH ($P < 0.01$), as was also observed for the pig [1], even when the experiments for rat, human and pig are performed under identical conditions. The reasons for the differences in K_A of the purified rat receptor for hCG, hLH and rLH are unknown. Many investigators using unpurified receptor preparations have found that affinity of the receptor for hCG is significantly higher than that for LH [35–37]. Studies using ovine luteal cells demonstrated that LH and hCG do not interact in an identical manner with the cells [38–40]. Importantly, the studies reported herein clearly demonstrate that the differences between LH and hCG in their interactions with the LH/hCG receptor are at least partly inherent to the purified receptor itself. It should be noted that LH/hCG receptors are exposed in the human ovary to both hCG and hLH in contrast to rat and pig LH receptors. In this context, there is no evidence that the human LH/hCG receptor is functionally desensitized *in vivo* during the first trimester of pregnancy when maternal hCG and progesterones are secreted simultaneously at high rates; furthermore, hCG administration *in vivo* did not densensitize the monkey corpus luteal LH/hCG receptor [41].

In summary, we have for the first time purified the human ovarian LH/hCG R to apparent homogeneity (based on one-dimensional SDS/PAGE) with the retention of selective hormone binding properties. Furthermore, we have demonstrated that the purified human, rat and porcine LH/hCG R may be a similar protein of a M_r 78,000 ± 2,000 and that the human receptor of M_r 78×10^3 can bind to hCG. The present studies also demonstrated that the interactions of the purified human receptor with hLH and hCG are markedly different from the interactions of hCG and hLH with purified rat and pig receptors.

After completion of this work, a cDNA for rat ovarian LH/hCG R was cloned [42], and the predicted amino acid sequence corresponds to a M_r of 75 kD protein. Simultaneously, the porcine testis LH/hCG R cDNA was cloned [43], and it also has a M_r of 75 kD. Thus, the predicted protein structure by itself has a M_r of 75 kD, and the predicted amino acid sequences of the receptor from the two species have 85% amino acid homology; however, the C terminal thirty amino acids show major differences. Since the human receptor M_r is identical to that of the pig and rat, but the human receptor has different hormone binding properties, it is possible that there could be subtle differences in conformation due to discrete differences in amino acids or in post-translational modifications which alter the interaction between the receptor and hormone in the human receptor as compared to those of the pig and rat. The differences in the proposed internal C terminal regions of the porcine and rat receptor may reflect functional differences between the rat and pig receptors and may explain differences in the stability of the receptor from the two species.

Acknowledgements—We thank the National Hormone and Pituitary Program for hormones, Dr. S. Saxena and Tom Veys for skillful assistance, and Glennora Flanagan for typing the manuscript. These studies were supported by N.I.H. Grant HD 21325 and American Cancer Society Grant PDT 329 awarded to Jayantha Wimalasena.

REFERENCES

1. Wimalasena J, Moore P, Wiebe JP, Abel JA and Chen TT, The porcine LH/hCG R. *J Biol Chem* **260**: 10689–10697, 1985.
2. Wimalasena J, Abel JA, Wiebe JP and Chen TT, The porcine ovarian luteinizing hormone/human chorionic gonadotropin receptor II. *J Biol Chem* **261**: 9416–9420, 1986.
3. Kusuda S and Dufau ML, Purification and characterization of the rat ovarian receptor for luteinizing hormone. *J Biol Chem* **261**: 16161–16168, 1986.
4. Minegishi T, Kusuda S and Dufau ML, Purification and characterization of Leydig cell luteinizing hormone receptor. *J Biol Chem* **262**: 17138–17143, 1987.
5. Kusuda S and Dufau ML, Characterization of ovarian gonadotropin receptor. *J Biol Chem* **263**: 3046–3049, 1988.
6. Keinanen KP, Kellokumpu S, Metsikko MK and Rajaniemi H, Purification and partial characterization of rat ovarian lutropin receptor. *J Biol Chem* **262**: 7920–7926, 1987.
7. Bruch RC, Thotakura NR and Bahl OP, The rat ovarian lutropin receptor. *J Biol Chem* **261**: 9450–9460, 1986.
8. Dattatreyamurty B, Rathman P and Saxena BB, Isolation of the luteinizing hormone-chorionic gonadotropin receptor in high yield from bovine corpora lutea. *J Biol Chem* **258**: 3140–3158, 1983.
9. Saxena BB, Dattatreyamurty B, Ota H, Milkov U and Ratnam P, Subunits of luteinizing hormone-human chorionic gonadotropin receptor from bovine corpora lutea. *Biochemistry* **25**: 7943–7950, 1986.
10. Lee CY, Coulum CB, Jiang NS and Ryan RJ, Receptors for human luteinizing hormone in human corpora luteal tissue. *J Clin Endocrinol Metab* **36**: 148–152, 1973.
11. Rao CV, Griffin LP and Carmen FR, Gonadotropin receptors in human corpora lutea of the menstrual cycle and pregnancy. *Am J Obstet Gynecol* **128**: 146–153, 1977.
12. Halme J, Ikonen M, Rutanen EM and Seppala M, Gonadotropin receptors of human corpus luteum during menstrual cycle and pregnancy. *Am J Obstet Gynecol* **131**: 728–734, 1978.
13. Wardlaw S, Lauersen NH and Saxena BB, The LH-hCG receptor of human ovary at the various stages of the menstrual cycle. *Acta Endocrinol (Copenh)* **79**: 568–576, 1975.
14. Rajaniemi HJ, Ronnberg L and Kauppila A, Luteinizing hormone receptors in human ovarian follicles and corpora lutea during menstrual cycle and pregnancy. *J Clin Endocrinol Metab* **52**: 307–313, 1981.
15. Keinanen KP and Rajaniemi HJ, Solubilization of luteinizing hormone receptor from human corpora lutea in a stable form and identification of the hormone-binding unit by ligand blotting. *J Clin Endocrinol Metab* **67**: 228–233, 1988.
16. Wimalasena J and Dufau ML, Gonadotropin receptors of the rat ovary. *Endocrinology* **110**: 1004–1012, 1981.
17. Dufau ML, Charreau EH and Catt KJ, Characteristics of a soluble gonadotropin receptor from the rat testes. *J Biol Chem* **148**: 6973–6982, 1973.
18. Laemmli UK, Cleavage of structural proteins during

the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
19. Wimalasena J, Indrapichate K, Wiebe JP, Abel JH and Chen TT, Luteinizing hormonal receptors of the mammalian ovary: Purification, characterization, and identification of the hormone binding subunit. In: *Membrane Proteins, Proceedings of Membrane Protein Symposium of 1986* (Ed. Goheen S), pp. 537–557. Bio-Rad Laboratories, Richmond, CA, 1987.
20. Bradford M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
21. Stoscheck CM, Protein assay sensitive at nanogram levels. *Anal Biochem* **160**: 301–305, 1987.
22. Shima K, Kitayama S and Nakano R, Gonadotropin binding sites in human ovarian follicles and corpora lutea during the menstrual cycle. *Obstet Gynecol* **69**: 800–806, 1987.
23. Meril CR, Goldman D and Van Keuren ML, Gel protein stains: Silver stain. *Methods Enzymol* **104**: 441–447, 1984.
24. Cerione RA, Strulovici B, Benovic JL, Lefkowitz RL and Caron MG, Pure β-adrenergic receptor: The single polypeptide confers catecholamine responsiveness to adenylate cyclase. *Nature* **306**: 562–566, 1983.
25. Einarson B, Gullick W, Conti-Tronconi B and Lindstrom J, Subunit composition of bovine muscle acetylcholine receptor. *Biochemistry* **21**: 5295–5302, 1982.
26. Ji I, Bock JH and Ji TH, Composition and peptide maps of cross-linked human choriogonadotropin–receptor complexes on porcine granulosa cells. *J Biol Chem* **260**: 12815–12821, 1985.
27. Ji I and Ji TH, Both A and B subunits of human choriogonadotropin photoaffinity label the hormone receptor. *Proc Natl Acad Sci USA* **78**: 5465–5469, 1981.
28. Ji I, Yoo BY, Kaltenbach C and Ji TH, Structure of the lutropin receptor on granulosa cells. *J Biol Chem* **256**: 10853–10858, 1981.
29. Rapporport B, Hazum E and Zor U, Photoaffinity labeling of human chorionic gonadotropin-binding sites in rat ovarian plasma membranes. *J Biol Chem* **259**: 4267–4271, 1984.
30. Rebois RV, Establishment of gonadotropin-responsive murine Leydig tumor cell line. *J Cell Biol* **94**: 70–76, 1982.
31. Rebois RV, Omedo-Sale F, Broady RO and Fishman P, Covalent crosslinking of human chorionic gonadotropin to its receptor in rat testes. *Proc Natl Acad Sci USA* **78**: 2086–2089, 1981.
32. Zhang Q-Y and Menon KMJ, Characterization of rat Leydig cell gonadotropin receptor structure by affinity cross-linking. *J Biol Chem* **263**: 1002–1007, 1988.
33. Hwang J and Menon KMJ, Characterization of the subunit structure of gonadotropin receptor in luteinized rat ovary. *J Biol Chem* **259**: 1978–1985, 1984.
34. Sojar HT and Bahl OP, Characterization of rat ovarian lutropin receptor. *J Biol Chem* **264**: 2552–2559, 1989.
35. Strickland TW and Puett D, Contribution of subunits to the function of luteinizing hormone/human chorionic gonadotropin recombinants. *Endocrinology* **109**: 1933–1942, 1981.
36. Rao ChV, Differential properties of human chorionic gonadotrophin and human luteinizing hormone binding to plasma membranes of bovine corpora lutea. *Acta Endocrinol (Copenh)* **90**: 696–710, 1979.
37. Huhtaniemi IT and Catt KJ, Differential binding affinities of rat testis luteinizing hormone (LH) receptors for human chorionic gonadotropin, human LH, and ovine LH. *Endocrinology* **108**: 1931–1938, 1981.
38. Niswender GD, Roess DA, Sawyer HR, Silvia WJ and Bayisas BG, Differences in the lateral mobility of receptors for luteinizing hormone (LH) in the luteal cell plasma membrane when occupied by ovine LH versus human chorionic gonadotropin. *Endocrinology* **116**: 164–169, 1985.
39. Mock EJ and Niswender GD, Differences in the rates of internalization of ^{125}I-labeled human chorionic gonadotropin, luteinizing hormone, and epidermal growth factor by ovine luteal cells. *Endocrinology* **113**: 259–264, 1983.
40. Mock EJ, Papkoff H and Niswender GD, Internalization of ovine luteinizing hormone/human chorionic gonadotropin recombinants: Differential effects of the A and B subunits. *Endocrinology* **113**: 265–269, 1983.
41. Ottobre JS and Stouffer RL, Receptors for chorionic gonadotropin in the corpus luteum of the rhesus monkey during simulated early pregnancy: Lack of down-regulation. *Endocrinology* **119**: 1594–1602, 1986.
42. McFarland KC, Sprengl R, Phillips HS, Kohler M, Nicolics K, Segaloft DL and Seeburg PH, Lutropin-choriogonadotropin receptor: An unusual member of the G protein-coupled receptor family. *Science* **245**: 494–499, 1989.
43. Loosfelt H, Misrahi M, Atger M, Salesse R, Thi MTVH, Jolivet A, Guiochon-Mantel A, Sar S, Jallal B, Garnier J and Milgrom E, Cloning and sequencing of porcine LH-hCG receptor cDNA: Variants lacking transmembrane domains. *Science* **245**: 525–528, 1989.