

# Pathways of Internalization of the hCG/LH Receptor: Immunoelectron Microscopic Studies in Leydig Cells and Transfected L-Cells

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**Abstract.** Monoclonal anti-receptor antibodies were used to study the cellular traffic of the hCG/LH receptor by immunoelectron microscopy. The LHR38 antibody was shown to bind to the extracellular domain of the receptor but not to interfere with hormone binding, adenylate cyclase activation or with the rate of internalization of the receptor. Pig Leydig cells and a permanent L-cell line expressing the LH receptor were used for the study. Incubation with LHR38-gold complexes showed the LH receptors to be randomly distributed over the cell surface including the clathrin coated pits. The LH receptors were internalized via a route including coated pits, coated vesicles and multivesicular bodies to lysosomes. This route is different from that observed for  $\beta$ -adrenergic, muscarinic, and yeast mating factor receptors and considered previously as possibly general for G-protein-coupled recep-

tors. The use of [ $^{125}$ I]LHR38 allowed precise measurement of the rate of internalization, showing the existence of a constitutive pathway which was increased 11-fold by hormone administration. Double labeling experiments suggested that the hormone (hCG-Au<sub>15nm</sub>) and the receptor (labeled with LHR38-Au<sub>5nm</sub>) have similar routes of endocytosis, both of them being degraded in lysosomes. Studies of the reappearance of LHR38-Au<sub>5nm</sub> on the surface of the cells and the use of monensin indicated that only a very small proportion of the receptor molecules were recycled to the cell surface. The distribution and the intracellular pathways of LH receptors are very similar in Leydig cells and transfected L-cells. This opens the possibility of using the latter to study, by *in vitro* mutagenesis, the molecular mechanisms involved in the cellular traffic of LH receptors.

CELL surface receptors for various ligands differ in their localization and their mechanisms and pathways of internalization. These receptors may be: (a) either specifically included in the coated pits (i.e., LDL-receptors [3, 4], transferrin receptors [18–20]); (b) expressed only outside the coated pits on the membrane (i.e.,  $\beta$ -adrenergic receptors) (37); or (c) randomly exposed on the cell surface, coated pits included (i.e., EGF-receptors) (11, 12). Under the effect of the ligand (or sometimes constitutively) (16, 46) the receptor is internalized and may follow one of the four major endocytic pathways which have been described: (a) the ligand-receptor complex dissociates at the endosomal level; the receptor is recycled to the surface whereas the ligand is degraded in lysosomes (i.e., LDL receptor) (7); (b) the ligand-receptor complex is recycled to the cell surface, dissociates and the receptor is reused (i.e., transferrin receptor) (20); (c) the ligand-receptor complex is delivered by transcytosis to the opposite front of polarized cells where the ligand is released intact and the receptor partially degraded (i.e., IgA receptor) (31); and (d) both partners are transported to lysosomes and degraded (i.e., EGF-receptor) (12).

The hCG/LH receptor is involved in the regulation of steroidogenesis. It has recently been cloned and sequenced (25, 30) showing it belongs to the group of seven transmembrane spanning G-protein linked receptors. However, contrary to previously studied receptors, it has a long NH<sub>2</sub>-terminal extracellular extension which is the site of its hormone binding function (48). Various indirect biochemical means have been used to study its internalization and down-regulation mechanisms (5, 6, 14, 15). However, due to the lack of adequate antibodies, no immunocytochemical studies of receptor cellular traffic have been reported. In general, few studies have been performed for the G-protein linked receptors with the exception of the muscarinic (36) and  $\beta$ -adrenergic (37) receptors. In the latter cases internalization has been reported to occur via smooth vesicles (36, 37) and this mechanism has been proposed for all G-protein-coupled receptors in general (41).

We have taken advantage of the preparation of specific monoclonal anti-LH receptor antibodies to study the distribution and internalization mechanisms of these receptors in Leydig cells. These cells, however, are not convenient for *in vitro* mutagenesis studies which will be necessary for a more

in depth analysis. Thus we compared LH receptor distribution and internalization in Leydig cells and in permanently transfected L-cells.

## Materials and Methods

### Chemicals

Special chemicals were obtained from the following sources: human chorionadotropin (hCG)<sup>1</sup>, iodination grade and [<sup>125</sup>I]hCG (sp. act. 50  $\mu$ Ci/ $\mu$ g), from UCB-Bioproducts (Braine-l'Alleud, Belgium); anti-mouse Ig-peroxidase complex, from Amersham International (Amersham, UK); sodium metaperiodate, trypsin inhibitor, isobutyl-methyl-xanthine, BSA, (fraction V), and adipic acid dihydrazide, from Sigma Chemical Co. (St. Louis, MO); collagenase-dispase from Boehringer Mannheim Biochemicals (Mannheim, Germany), streptomycin, penicillin and gentamycin from Gibco Laboratories (Grand Island, NY), osmium tetroxide, paraformaldehyde and glutaraldehyde for EM from Merck (Darmstadt, Germany); Poly/Bed 812 embedding media, nadic methyl-anhydride, dodecylsuccinic anhydride, and 2,4,6-tri(dimethylamino-methyl)phenol from Polysciences, Inc. (Warrington, PA); and colloidal gold solutions from Bio Cell Research Laboratories (Cardiff, UK).

Purified porcine hCG/LH-receptor and mouse monoclonal anti-porcine hCG/LH-receptor antibody 38(LHR38) were obtained and characterized as previously described (44).

### Tracers

**Gold Conjugates.** LHR38-Au<sub>5nm</sub>, IDA10-Au<sub>5nm</sub>, and hCG-Au<sub>15nm</sub> complexes were prepared according to standard methods (10). For cell incubation the gold complexes were resuspended in Dulbecco's PBS containing 1% BSA to a final concentration corresponding to  $A_{520nm}^{500nm} = 0.1$  for LHR38-Au<sub>5nm</sub> and IDA10-Au<sub>5nm</sub>, and  $A_{520nm}^{500nm} = 0.3$  for hCG-Au<sub>15nm</sub>. By EM the gold conjugates were monodisperse.

**Radiolabeled LHR38.** LHR38 was radiolabeled by using Enzymo-Bead radioiodination reagent and the procedure recommended by BioRad (Richmond, CA). The specific radioactivity obtained was 1.2  $\mu$ Ci/ $\mu$ g of protein.

### Leydig Cell Preparation

Porcine testes were obtained by castration of 20-d-old piglets. Isolated Leydig cells were prepared by collagenase-dispase digestion followed by a Percoll gradient purification (29). The cells were plated and cultured for 6 d on plastic dishes in a 1:1 mixture of DME and HAM F12 supplemented with 2% Ultrosor G, $\alpha$ -tocopherol (0.2 ng/ml), 10 U/ml streptomycin, 10 U/ml gentamycin, and 100 U/ml penicillin.

### Generation and Culture of Transfected L-cell Line Expressing the LH Receptor

An immortalized line of transfected L-cells was obtained by using an expression vector encoding the full length porcine LH receptor. This vector contains the cytomegalovirus promoter pc DNA<sub>1</sub>, Invitrogen. It was co-transfected with a selection vector for the resistance gene of geneticin (39). The resistant cells were cloned by limiting dilution and a clone expressing high levels of LH receptor was selected by ligand binding assay and immunocytochemistry.

### Solid Phase Assay for Acid-dependent Release of LHR38-Au<sub>5nm</sub> from Purified Porcine LH Receptor

Assays were performed in Nunc-Immuno-Plates, MaxiSorp 96 (Nunc, Roskilde, Denmark). Purified ovarian LH receptor was absorbed to each well by incubation at a final concentration of 3  $\mu$ g/ml in 100  $\mu$ l of Tris-HCl buffer, pH 8.5 containing 2 mM CaCl<sub>2</sub>. After 16 h at 4°C, the solution was removed. Nonspecific binding sites were saturated by filling the wells completely with PBS, pH 7.4, containing 0.1% Tween 20, and 1% BSA and incubating them at room temperature for 1 h. The wells were then washed four times with PBS containing 0.1% Tween 20 and 0.1% BSA, and a solu-

tion containing either LHR38-Au<sub>5nm</sub> (at a concentration corresponding to  $A_{520nm}^{500nm} = 0.1$ ) or free LHR38 (3  $\mu$ g/ml) was added in a volume of 75  $\mu$ l. After 60 min at room temperature the excess marker was washed out with PBS. To determine the pH value at which the dissociation of receptor-monoclonal anti-receptor antibody complex occurs, the wells were washed twice with PBS-BSA adjusted at various pH values with HCl. The proportion of antibody still associated with the receptor was determined with anti-mouse Ig-peroxidase complex by using the ABTS substrate and the procedure recommended by Amersham International (Amersham, UK).

### Radiolabeling Studies

For binding and internalization studies the cells were plated on 3.5-cm tissue culture dishes and 24-well plates (Costar) and grown in DME (DME) supplemented with 10% FCS and for transfected L-cells with 0.04% geneticin. "Confluent" cells were incubated with [<sup>125</sup>I]LHR38 in PBS supplemented with 0.1% BSA (PBS-BSA). At the end of incubations, the cells were washed with appropriate buffer solutions and dissolved in 0.2 M NaOH. Radioactive counts were performed with a gamma counter.

### BINDING ASSAYS

**Time-dependence of LHR38 Binding.** This was determined by incubating cells with [<sup>125</sup>I]LHR38 in PBS-BSA for periods of 5, 10, 20, 30, and 60 min at 4°C. After three washes with PBS the radioactivity associated with the cells was counted.

**Saturability.** The cells were incubated for 30 min at 4°C with increasing concentrations (from 3 to 270 nM) of [<sup>125</sup>I]LHR38. The nonsaturable nonspecific ligand binding was determined by parallel incubations in the presence of a 150-fold excess of unlabeled LHR38. The specific binding was calculated by subtracting nonspecific binding from the total binding. The data for specific binding were then used to construct a Scatchard plot which allowed to estimate the number of binding sites per cell.

### INTERNALIZATION ASSAYS

Cells grown on 3.5-cm tissue culture dishes were rinsed twice with PBS-BSA at 4°C and preincubated with 20 nM [<sup>125</sup>I]LHR38 for 30 min. By immersing the plates in a water bath at 37°C the cells were then incubated for various periods of time in the presence or absence of hCG (2.5  $\mu$ g/ml). At the end of the incubations the excess ligand was washed out with PBS-BSA. The surface-bound ligand was washed away with PBS-BSA adjusted to pH 2 with HCl (3 min at 4°C). The ligand remaining associated to the cells after the wash at pH2 was considered as internalized.

Where indicated, cells were preincubated at 37°C for 30 min with 50  $\mu$ M monensin in PBS-BSA.

### Determination of Cyclic AMP

The cyclic AMP produced by Leydig cells after their stimulation with various ligands (Table I) was determined by using the procedure and the cyclic AMP [<sup>3</sup>H] assay system (code TRK 432) (Amersham, UK). Butyl-methyl-xanthine (0.5 mM) was used as phosphodiesterase inhibitor.

### Down Regulation of LH-hCG-R

Both cultured Leydig cells and transfected L-cells were incubated at 37°C for 60 min with: (a) IDA<sub>10</sub> (a mAb used as a control (23) (10  $\mu$ g/ml); (b) LHR38 (10  $\mu$ g/ml); (c) LHR38-Au<sub>5nm</sub> ( $A_{520nm}^{500nm} = 0.1$ ); (d) hCG (2.5  $\mu$ g/ml); and (e) LHR38-Au<sub>5nm</sub> ( $A_{520nm}^{500nm} = 0.1$ ) + hCG (2.5  $\mu$ g/ml). After two washes with PBS, the cells were washed with PBS-BSA at pH 2 (3  $\times$  1 min) to remove the cell surface-associated ligand. Then the cells were neutralized with 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, washed three times (3  $\times$  1 min) with PBS-BSA and incubated at 4°C for 60 min with 60 nM of [<sup>125</sup>I]hCG. The non-specific [<sup>125</sup>I]hCG binding was determined in the presence of 6  $\mu$ M of unlabeled hCG. The unbound radiolabeled hormone was washed out with PBS (3  $\times$  2 min). Finally, the cells were treated with 0.2 M NaOH and the radioactivity counted. Cells receiving PBS instead of ligand and L-cells lacking LH receptor were used as a control.

### Light Microscopy

Transfected L-cells (permeabilized with acetone at -70°C) were incubated at room temperature for 60 min with LHR38-Au<sub>5nm</sub> complex ( $A_{520nm}^{500nm} = 0.1$ ) in PBS containing 1% BSA. The unbound marker was washed out with

1. **Abbreviations used in this paper:** hCG, human chorionadotropin; LH, lutropin hormone; LDL, low density lipoprotein.

PBS (2 × 1 min) and distilled water (5 × 1 min). The immunogold staining was intensified by using Silver Enhancement reagents (BioCell, Cardiff, UK). L-cells lacking LH receptors were used as control.

## EM

### DISTRIBUTION OF LH RECEPTOR ON THE CELL SURFACE

Three different experimental conditions were used:

**Detection of LH Receptors on Fixed Cells.** Cells were fixed at room temperature for 15 min with a fixative mixture containing 2% paraformaldehyde, 0.1 M adipic acid dihydrazide, 0.01 M sodium metaperiodate, and 0.05 M phosphate buffer, pH 7.3. All subsequent steps, unless otherwise specified, were performed at room temperature. The residual aldehyde groups were quenched with 0.05 M NH<sub>4</sub>Cl (27) in PBS (three changes over 15 min). The cells were treated with 1% BSA in PBS for 30 min and then incubated 60 min with either LHR38-Au<sub>5nm</sub> or IDA 10-Au<sub>5nm</sub> (control antibody) complexes. The unbound markers were washed out with PBS-BSA (3 × 10 min each) and the cells were further fixed for 30 min with 2.5% glutaraldehyde and 5% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, and finally processed for EM (see below). The specificity of the binding was established by addition of unlabeled LHR38 at a final concentration of 100 μg/ml PBS-BSA.

**Detection of LH Receptors on Unfixed Cells.** The cells were rinsed twice (3 min each) at room temperature and once at 4°C (5 min) with PBS-BSA, and incubated with LHR 38-Au<sub>5nm</sub> for 30 min at 4°C. Then the cell layers were rinsed three times (2 min each) at 4°C with PBS and fixed at 4°C for 30 min with 2.5% glutaraldehyde and 5% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, and finally processed for EM.

**Cell Surface Distribution of LH Receptors after Incubation with hCG or with LHR38-Au<sub>5nm</sub>.** Petri dishes containing either Leydig cells or transfected L-cells were rinsed 2 × 3 min at room temperature and once (5 min) at 4°C with PBS-BSA and incubated with LHR38-Au<sub>5nm</sub> for 30 min at 4°C. After three washings with cold (4°C) PBS-BSA the cultures were incubated for 5 min at 37°C in PBS-BSA with or without addition of 2.5 μg hCG/ml, washed with PBS, fixed, and processed for EM.

### RECEPTOR TRAFFICKING FOLLOWED WITH LHR38-AU<sub>5nm</sub> COMPLEX

The experiments were carried out as described for internalization radioassays (see above) replacing the radioiodinated antibody with LHR38-Au<sub>5nm</sub> complex at a concentration corresponding to A<sub>540nm</sub><sup>300</sup> = 0.1. After the appropriate time of incubation at 37°C in the absence or presence of the hormone (2.5 μg hCG/ml) the cells were rinsed twice with PBS, fixed for 30 min at room temperature with 2.5% glutaraldehyde and 5% paraformaldehyde in 0.1 M cacodylate buffer and prepared for EM.

### DOUBLE LABELING EXPERIMENTS

After two washes at room temperature and once at 4°C with PBS-BSA the cells were incubated at 4°C for 30 min with a mixture of LHR38-Au<sub>5nm</sub> and hCG-Au<sub>15nm</sub> complexes in PBS-BSA at a concentration corresponding to A<sub>540nm</sub><sup>300</sup> = 0.1 and A<sub>540nm</sub><sup>1500</sup> = 0.3, respectively. The cells were then rinsed twice with PBS-BSA at 4°C, warmed to 37°C by immersion of the plates in a water bath and incubated for 2, 5, 10, 15, 30, and 60 min in the absence of ligands. Where indicated after 5 and 10 min of exposure to the tracers the cells were washed at 4°C with PBS-BSA adjusted at pH 2 with HCl (3 × 1 min), neutralized with 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, and then warmed by incubating in buffered medium at 37°C for 15 min in the absence of the tracers. After the incubation periods, the cells were washed with PBS and fixed (30 min at room temperature) with 2.5% glutaraldehyde and 5% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, and processed for EM.

### Processing for EM

After the fixing step (see above) the cells were postfixed in 1% OsO<sub>4</sub> in 0.1 M cacodylate buffer, pH 7.3 (45 min at 4°C), stained with 0.5% uranyl acetate (15 min; room temperature), and dehydrated in ethanol. Cell monolayers released from the polystyrene dishes with propylene oxide were pelleted by centrifugation and embedded in Epon 812. Sections were cut on a Ultracut microtome (Reichert Jung S. A., Paris), stained with uranyl

acetate and lead citrate and examined at 80 kV in an Elmiscop 101 electron microscope (Siemens Corp., Iselin, NJ).

## Quantification of the Distribution of LH Receptor on Different Domains of the Cell Surface

Cell profiles (typically 45–50 for each situation described above) randomly selected were photographed at 30,000 magnification and the film negatives were used for quantification.

Morphometric analysis was performed with a SAMBA 2005 ALCATEL-TITN image analyzer. This system comprised a VIDICON PANASONIC video camera connected to a MATROX MVP NP video digitalized card and a COMPAQ DESKPRO 386 S microcomputer. Each film negative read by the video camera was digitized into 512 × 512 pixels each with a 256 grey level value. The operator screen drew the cell perimeters using a mouse and marked the coated pits, smooth vesicles and plasmalemma proper by cutting the plasma membrane with a rubber. These domains were automatically labeled and extracted by the operator. At the end of the analysis the computer displayed the total length of plasma membrane and the relative length of each plasma membrane domain. The total length of measured plasma membrane was 125, 129, and 118 μm for fixed, non-stimulated, and stimulated Leydig cells, respectively. For transfected L-cells the total length of examined plasma membrane was 158 μm for fixed cells, 170 μm for non-stimulated cells, and 152 μm for stimulated cells. The number of gold particles associated with each plasma membrane domain were counted and the results were expressed as percentage of the total number of particles (~500 for each experiment) associated with the plasma membrane.

## Results

### LHR38 Anti-receptor Antibody Is Suitable for the Study of LH Receptor Distribution and Internalization

Several properties of the mAb LHR38 either in free form or complexed to gold particles had to be verified before using it as a marker for the LH receptor in electron-microscopic studies. It was necessary that the antibody be neutral in terms of receptor activation or internalization. Leydig cells were thus incubated with LHR38, LHR38-Au, a control non-receptor related antibody, and hCG alone or in combination with LHR38 (free or tagged with gold). Measurements of cyclic AMP production (Table I) showed the absence of any effect of LHR38.

Receptor down-regulation was examined in both Leydig cells and Transfected L-cells similarly incubated with antibody or hormone. As illustrated in Table II while hCG induced in 60 min at 37°C about a 40% decrease in [<sup>125</sup>I]hCG binding, no down-regulation of LH receptor was observed

Table I. Effect of LHR38 Antibody and of Hormone on cAMP Production in Leydig Cells

Ligand	Concentration	pmoles of cAMP/10 <sup>6</sup> cells		
		5 min	15 min	30 min
	(M)			
PBS (control)	—	33	34	53
LHR38	7.10 <sup>-9</sup>	44	50	59
LHR38-Au <sub>5nm</sub>	A <sub>540nm</sub> <sup>300</sup> = 0.1	40	44	52
hCG	7.10 <sup>-9</sup>	621	1,284	1,800
hCG + LHR38	7.10 <sup>-9</sup>	760	1,281	2,080
hCG + LHR38-Au <sub>5nm</sub>	7.10 <sup>-9</sup>	760	1,184	1,896
	A <sub>540nm</sub> <sup>300</sup> = 0.1			

**Table II. Effect of LHR38 Antibody and of Hormone on the Down-regulation of the LH Receptor in Leydig Cells and L Cells\***

Incubation in presence of	Bound [ <sup>125</sup> I]hCG (c.p.m./10 <sup>6</sup> cells)†		
	Leydig cells	Transfected L-cells	Non-transfected L-cells
PBS-BSA	20,810 + 480	595,100 + 2,300	260 + 100
Control antibody, IDA10	20,700 + 450	592,100 + 4,100	254 + 130
LHR38 antibody	20,300 + 600	585,200 + 6,300	259 + 145
LHR38-Au <sub>5nm</sub>	20,400 + 530	586,560 + 4,570	258 + 160
hCG	8,860 + 530	236,100 + 1,470	240 + 180
LHR38-Au <sub>5nm</sub> + hCG	7,830 + 700	226,600 + 2,100	257 + 145

\* The cells were incubated with various ligands for 60 min at 37°C. After washing with PBS the cell surface associated ligands were removed with a PBS-BSA, pH 2, buffer. The receptors remaining on the cell surface were quantified by incubating the cells with 60 nM of radiolabeled hormone (sp. act.: 50 μCi/μg). The non-specific [<sup>125</sup>I]hCG binding was determined in the presence of 6 μM of unlabeled hCG and subtracted.

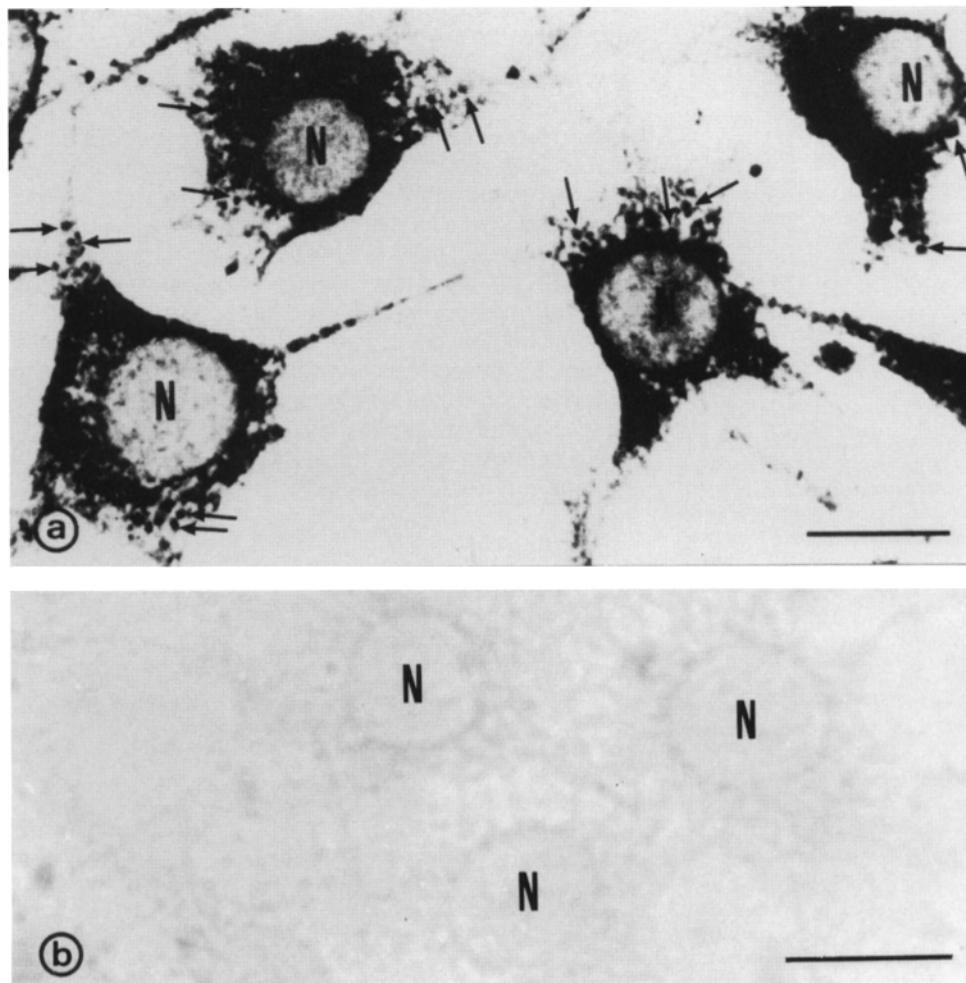
† The data are expressed as mean + SD (n = 3).

‡ The concentrations used were: control antibody, IDA10 (10 μg/ml); LHR38 (10 μg/ml); hCG (2.5 μg/ml); LHR38-Au<sub>5nm</sub> (at a concentration corresponding to A<sub>500nm</sub> = 0.1).

when the cells were incubated with free or gold complexed LHR38.

Since low pH exists in several intracellular structures we also examined the stability of LH receptor-LHR38 complexes. Purified LH receptor was adsorbed onto wells of Nunc-Immunoplates and incubated with either free LHR38

or LHR38-Au<sub>5nm</sub>. Washings were performed at various pH values and remaining antibody measured with anti-mouse Ig-peroxidase complex. Washings with buffers of pH 7.4 to 3.5 did not remove significant (<10%) amounts of the antibody complexed to the receptor. However, at pH ≤3 the receptor-antibody complex dissociated, 95% of the anti-



**Figure 1.** (a) Transfected L-cells expressing the LH receptor: receptor localization in light microscopy with LHR38-Au<sub>5nm</sub> after cell permeabilization with acetone. The immunogold staining was intensified with Silver Enhancement reagents from bioCell. The punctuated staining (arrows) suggests the presence of LH receptors in endosomes/lysosomes. (b) Control cells (non-transfected L-cells) processed as in a. Note the absence of reaction with anti LH receptor antibody. N, nucleus. Bar, 10 μm.

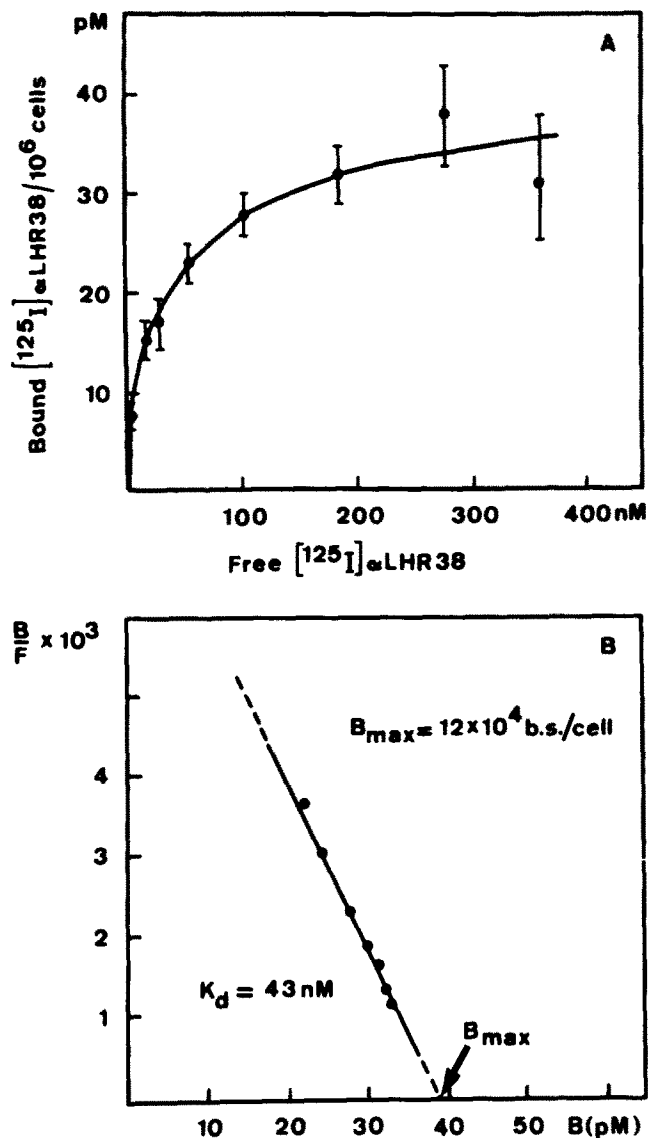


Figure 2. Saturation analysis of [<sup>125</sup>I]LHR38 binding to transfected L-cells expressing the LH receptor. The cells were incubated at 4°C for 30 min with increasing concentrations of [<sup>125</sup>I]LHR38 (1.2 μCi/μg protein). (A) The [<sup>125</sup>I]LHR38 bound to the cells was measured. (B) A Scatchard plot was constructed with the data presented in A.

body being released at pH 2. Hence, receptor-antibody complex should have been preserved in the intracellular structures (lowest pH 4.5 for lysosomes) (33).

#### Characterization of a Permanent L-cell Line Expressing the LH Receptor

The transformed L-cells were fixed and permeabilized at -70°C with acetone and incubated with the LHR38-Au<sub>5nm</sub> complex. Silver enhancement and light microscopy were used. The immunocytochemical reaction showed all the cells to express the receptor. The staining of the cytoplasmic areas was rather diffuse probably due to the silver enhancement procedure. However, a punctuated staining of the cells (Fig. 1 a, arrows) suggesting the presence of LH receptors in endosomes/lysosomes was observed. There was no labeling of the nucleus. The immunocytochemical reaction was abolished by the addition of a high concentration (100 μg/ml) of

unlabeled LHR38. No binding of LHR38-Au<sub>5nm</sub> was observed on control cells (non-transfected L-cells) (Fig. 1 b).

When the cells were incubated at 4°C for different time intervals with [<sup>125</sup>I]LHR38, the binding reached a steady-state plateau at 30 min (this time of incubation was used for all further experiments). The binding of LHR38 to the cell surface was saturated at a concentration of 30 nM of radiolabeled monoclonal anti-receptor antibody. The profile of the saturation curve is illustrated in Fig. 2 A. At all concentrations used the nonspecific binding was low (<7% of the total binding). Scatchard analysis (Fig. 2 B) of the data for specific binding gave a cell surface receptor concentration of  $1.2 \times 10^4$  binding sites/cell, and a dissociation constant of receptor-antibody complexes of  $K_D = 43$  nM.

#### Cell Surface Distribution of the hCG/LH Receptor

The initial distribution of LH receptors was examined on the surface of non-hormone stimulated cells. Leydig cells and transfected L-cells were either initially fixed and then exposed to antibody-gold complexes or incubated with LHR38-Au<sub>5nm</sub> for 30 min at 4°C, washed, and then fixed and processed for EM. The decoration pattern was examined on the plasmalemma proper, the coated pits and the membrane of smooth vesicles open to the culture medium. The relative length of these plasma membrane domains is given for each cell type in Table III.

As a general feature, irrespective of condition used (initial fixing or fixing after hormone incubation) both Leydig cells and transfected L-cells had comparable decoration patterns with LH receptors distributed diffusely over the plasmalemma proper and coated pits. In all cases the marker failed to label the smooth vesicles open to the culture medium (virtually no particles were observed associated with these structures out of 1,500 gold particles counted) (Figs. 3 and 4). Examined in more detail, the distribution of LH receptors had some specific characteristics for each cell type (Table III). Thus, in Leydig cells ~85% of LH receptors were associated with plasmalemma proper and ~15% with the coated pits. On the other hand, in transfected L-cells ~25% of the LH receptors were present in the coated pits, the rest of the receptors being associated with plasmalemma proper. When the living cells were warmed to 37°C this decoration pattern did not change significantly up to 5 min (Table III). These observations suggest that the antibody-gold complex did not induce clustering of receptor molecules.

After stimulation of the cells with hCG at 37°C the LH receptors became clustered (a process more evident in transfected L-cells (Fig. 4, inset) and subsequently concentrated in coated pits (Table III). Using the data from Table III we have determined (as described by Hansen et al. in reference 17) the concentration efficiency of LH receptors (it is given by the ratio of [% gold in coated pits]/[% of perimeter occupied by coated pits]/[1-% gold in coated pits]/[1-% of plasma membrane occupied by coated pits]). In non-stimulated Leydig cells the concentration efficiency of LH receptors was of 8.4, a value that was increased 9.6-fold by hormone administration. For transfected L-cells the concentration efficiency was of 21.8, a value that was increased 8.6-fold in the presence of hCG.

#### Pathways of Internalization of the LH Receptor

Incubation of Leydig cells with LHR38-Au<sub>5nm</sub> in the absence of hormone showed that the receptor molecules are in-

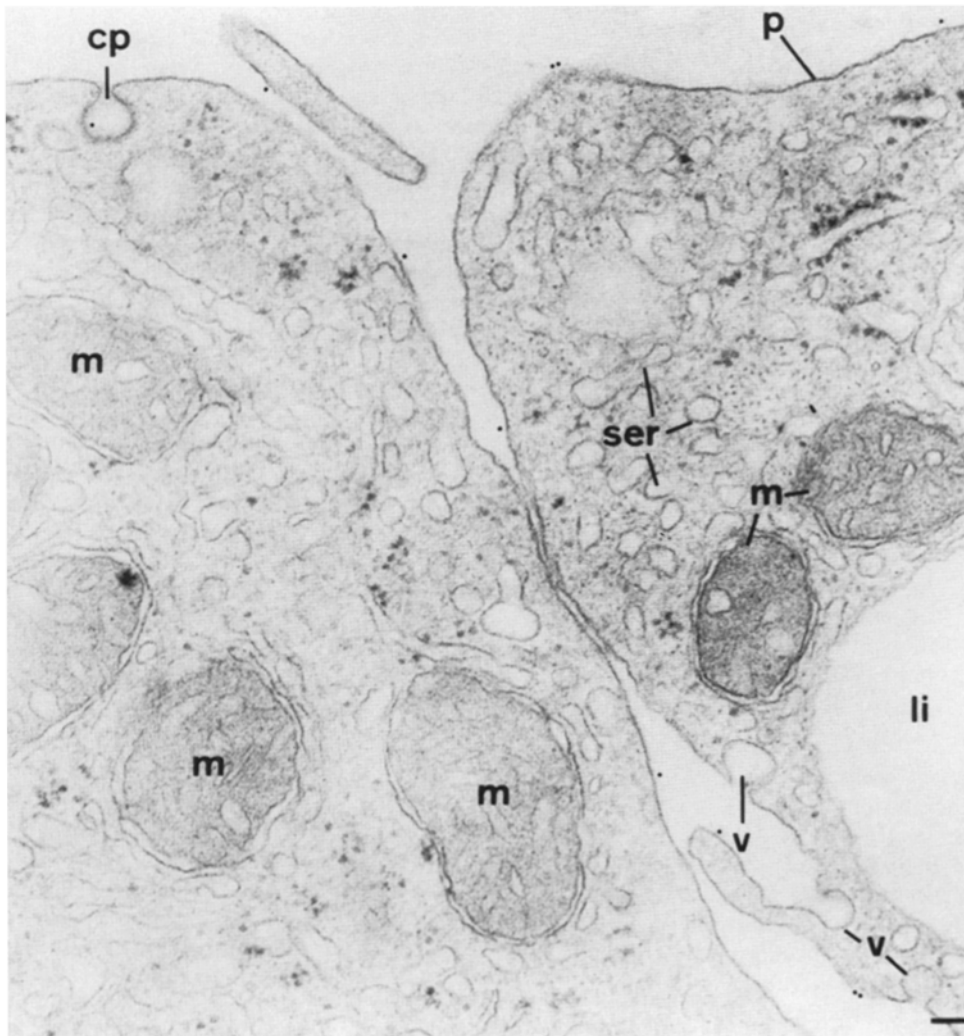
**Table III. Labeling by LHR38-Au<sub>5nm</sub> of Plasma Membrane Domains of Leydig Cells and of L-cells Expressing the LH Receptor**

	Leydig cells			Transfected L-cells		
	cp	sv	pp	cp	sv	pp
	(%)					
Relative area of plasma membrane domains*	2.3	9.4	88.3	1.8	11.3	86.9
LHR38-Au particles associated with plasma membrane domains						
fixed cells	15.1	0	84.9	25.3	0	74.7
non-fixed cells						
unstimulated	16.5	0	83.5	28.6	0	71.4
hCG stimulated	65.5	0	34.5	77.4	0	22.6

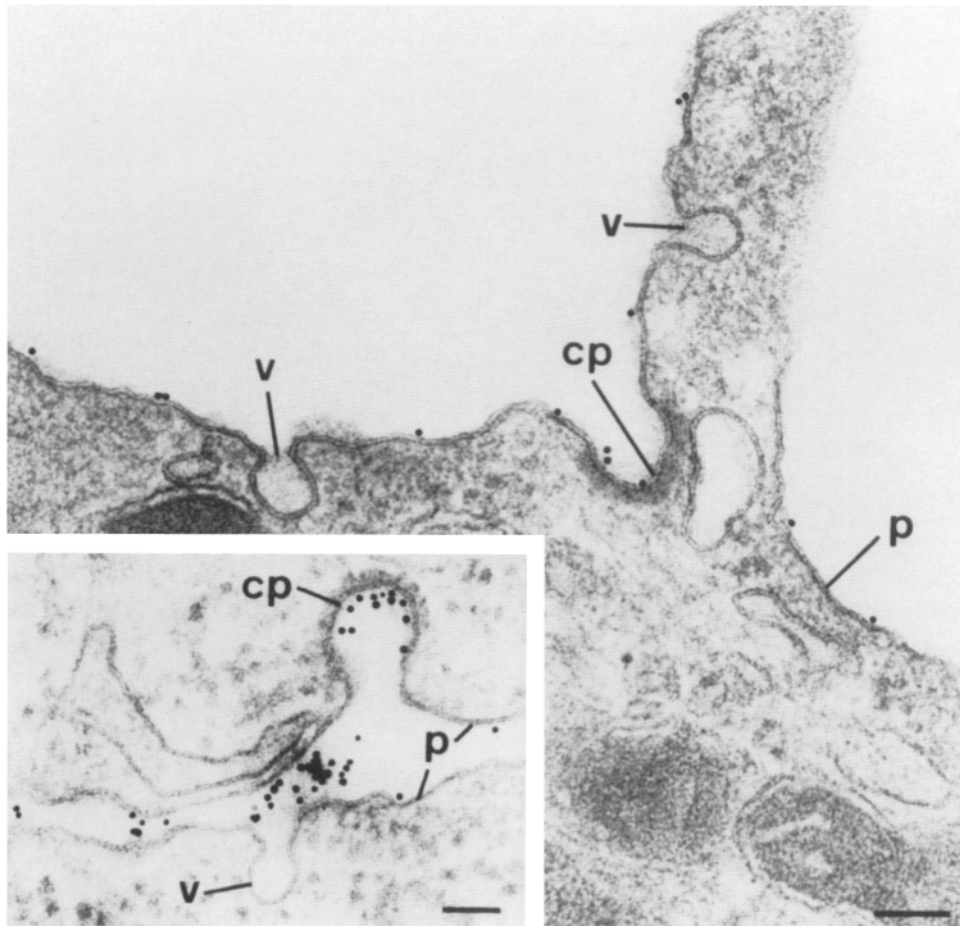
\* Identical results were observed in hCG stimulated cells and in unstimulated cells.

cp, coated pits; pp, plasmalemma proper; sv, smooth vesicles. (For experimental details see the Materials and Methods chapter).

ternalized via coated pits and vesicles (5 min), endosomes (5–15 min), multivesicular bodies (15–30 min) to lysosomes (after 30 min). Increased labeling of all these structures was observed when the cells received hCG (2.5 µg/ml) (Fig. 5). At the level of multivesicular bodies, the receptor molecules were associated with vesicles. This has been considered (12) as an indication of targeting to lysosomes. In absence of hormone the number of receptor molecules which are detected in multivesicular bodies (Fig. 5 b) and in lysosomes (Fig. 5 c) is limited. We also incubated the transfected L-cells with LHR38-Au<sub>5nm</sub> in the presence of hormone. The internalization pattern (Fig. 6) was very similar to that observed in the Leydig cells. After 2 to 5 min of exposure with antibody-gold conjugate, the tracer particles were localized in coated pits (Fig. 6 a), in coated vesicles (Fig. 6 b) and in small tubulovesicular structures of the endosomal compartment (Fig. 6 c). After 10 to 30 min the gold particles were localized to multivesicular bodies (largely associated with internal vesicles) (Fig. 6 d) and vacuoles of lysosomal appearance (the latter generally after 30 min) concentrated in the juxtannuclear area (Fig. 6 e). The lysosomes were also identified by the presence of acid phosphatase (not shown).



**Figure 3.** Cell surface distribution of LH receptors in non-hormone-stimulated Leydig cells. After 30-min incubation at 4°C with LHR38-Au<sub>5nm</sub> and washing of unbound marker, the cells were warmed for 5 min at 37°C in absence of hormone. Note that the marker does not induce clustering of LH receptors associated with plasmalemma proper (p) and coated pits (cp). The gold particles fail to label the smooth vesicles (v) open to the culture medium. A similar decoration pattern was obtained when the cells were fixed and then incubated with antibody-gold complex (not shown). li, lipid inclusion; m, mitochondrium; ser, smooth endoplasmic reticulum. Bar, 0.1 µm.



**Figure 4.** Cell surface distribution of LH receptors in a L-cell line expressing the LH receptor. The cells were incubated with LHR38-Au<sub>5nm</sub> for 30 min at 4°C, washed, and then incubated for 5 min at 37°C in the absence of hCG. As in the case of Leydig cells, the gold particles mark the plasmalemma proper (*p*) and coated pits (*cp*). No binding is observed on smooth vesicles (*v*) open to the culture medium. *Inset:* distribution of LHR38-Au<sub>5nm</sub> in hormone-stimulated cells. Note the clustering of receptor on the plasmalemma proper (*p*), the internalization of aggregated receptors via coated pits (*cp*), and the absence of gold particles in a smooth vesicle (*v*) open to the culture medium. Bar, 0.1 μm.

To precisely quantify the rate of internalization of the receptor in the absence or presence of hormone we incubated the transfected L-cells with [<sup>125</sup>I]LHR38 at 37°C. In the absence of hCG, 5% of the cell associated radioactivity was internalized 30 min after the beginning of the incubation. This constitutive receptor internalization was increased 11-fold by the action of the hormone and reached a steady-state plateau at 20-30 min (see Fig. 8).

#### **Hormone and Receptor Molecules Have Similar Pathways of Internalization**

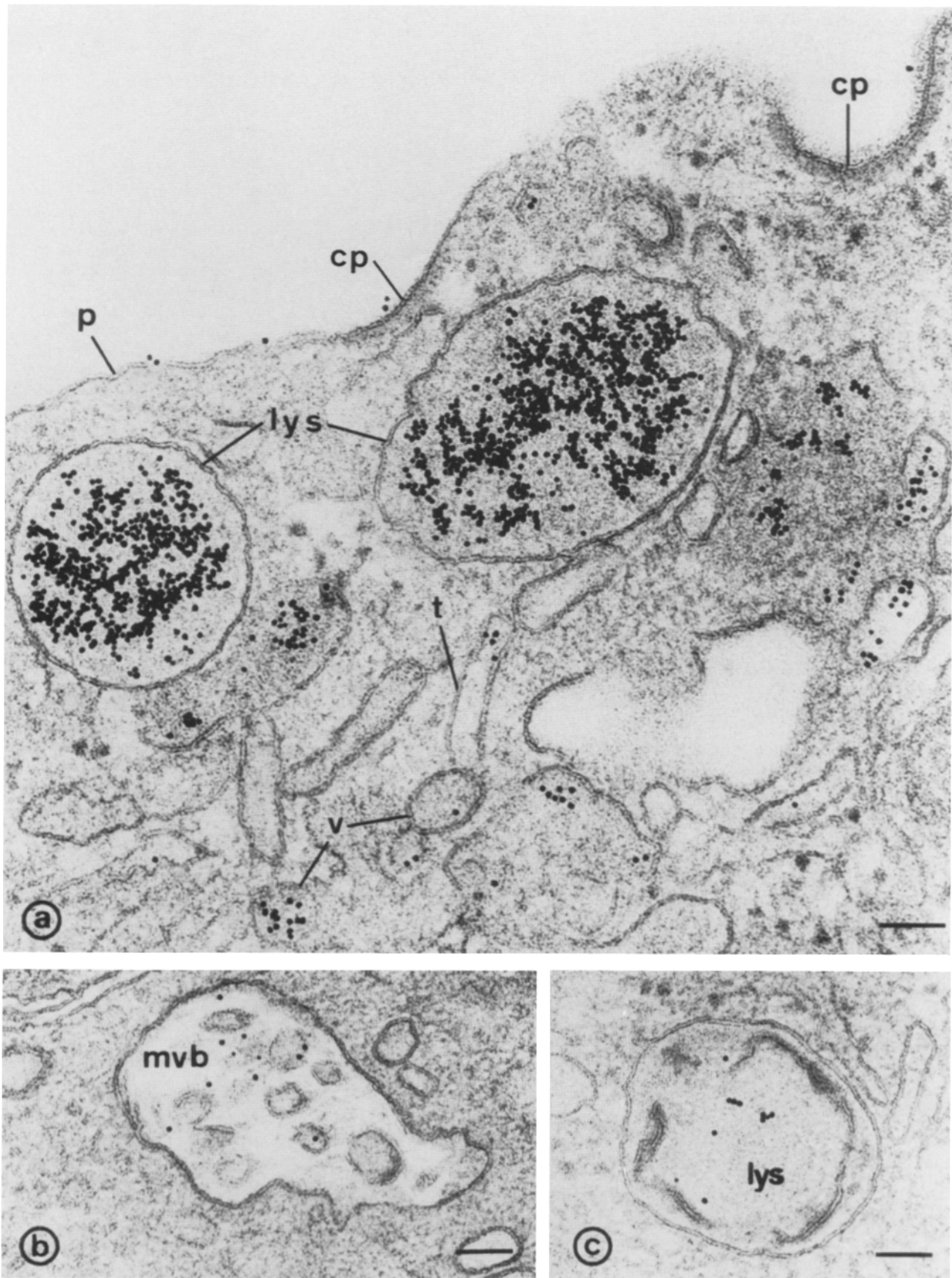
To follow ultrastructurally the intracellular traffic of both hCG and its receptor to various compartments involved in the endocytic pathway, we incubated Leydig cells with a mixture of tracers consisting of LHR38-Au<sub>5nm</sub> (for detecting receptor molecules) and hCG-Au<sub>15nm</sub> (to follow the hormone). The results of this double morphological study are illustrated in Fig. 7. The hormone and receptor molecules were colocalized on the plasma membrane (Fig. 7 *a*), in coated pits and vesicles (Fig. 7, *b* and *c*), in the small tubulovesicular structures of the endosomal compartment, in the multivesicular bodies (Fig. 7 *d*), and in the lysosomes (Fig. 7 *e*).

#### **A Very Small Proportion of LH Receptor Is Recycled**

Receptors residing at the cell surface were tagged specifically with LHR38-Au<sub>5nm</sub> and/or hCG-Au<sub>15nm</sub> at 4°C for 30

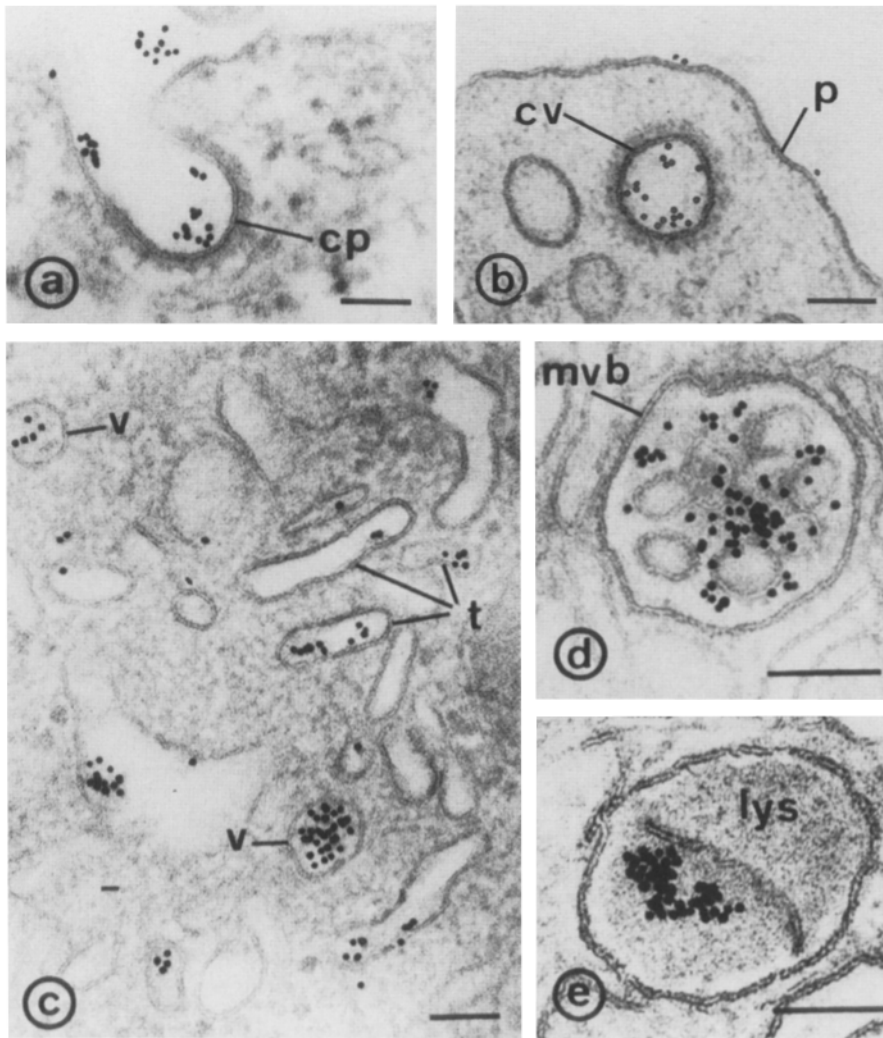
min and allowed to be internalized by warming for 5- or 10-min intervals at 37°C. The cells were then washed with PBS, treated at 4°C with PBS-BSA of pH2 (3 × 1 min) to dissociate antibody and hormone from the cell surface receptors and neutralized with 0.2 M Na<sub>2</sub>HPO<sub>4</sub>. The cells were finally incubated for 15 min in culture medium to allow the recycling of the receptor. The eventual reappearance of the gold particles on the cell surface was considered representative of recycled receptors. The results of these experiments (not illustrated) indicated that only a small proportion (<1%) of the internalized receptors were returned to the cell surface. Because both receptor -(LHR38-Au<sub>5nm</sub>) and receptor-hCG complexes are stable at pH ≥4.5 (the pH values of the endocytic system), we concluded that in our conditions and within the limits of this experimental system, recycling of LH receptor is insignificant.

Another approach used monensin to study receptor recycling. This drug inhibits both the synthesis of receptors and the return of internalized membrane receptors to the cell surface (7, 8, 12, 16, 40, 47). We thus compared the accumulation of [<sup>125</sup>I]LHR38 inside the cells in the absence or in the presence of the drug. If recycling was present, cells incubated with monensin should have had markedly increased intracellular receptor compared to controls. As shown in Fig. 8 this was not the case, only a small increase (7% of control) was observed. This method thus also indicates a very limited degree of recycling of previously internalized receptor.



**Figure 5.** Leydig cells in culture: Internalization of the hCG/LH receptor (visualized with LHR38-Au<sub>5nm</sub> complex) after 60-min incubation at 37°C in the presence (a) or absence (b and c) of hormone (2.5 μg hCG/ml). (a) The marker labels the plasmalemma proper (p), coated pits (cp), vesicular (v), and tubular (t) endosomal structures and lysosomes (lys). (b) Multivesicular body (mvb) studded with LHR38-Au complex. Note receptor association with internal vesicles. (c) Lysosome containing a small number of receptors internalized in the absence of hormone (compare with lysosome labeling in a). Bar, 0.1 μm.





**Figure 6.** An L-cell line expressing the LH receptor: steps in endocytic internalization of the receptor in presence of 2.5  $\mu\text{g}$  hCG/ml. The cells were incubated with LHR38-Au<sub>5nm</sub> for 30 min at 4°C, washed, and warmed at 37°C for 5 min (a and b), 15 min (c), 30 min (d), and 60 min (e). (a and b) Internalization via coated pits (cp) and coated vesicles (cv). (c) Vesicular and tubular structures of the endosomal compartment containing the internalized receptor. (d) Multivesicular body (mvb); (e) lysosome (lys) studded with tracer particles. The presence of marker particles in lysosomes suggests receptor degradation. Bar, 0.1  $\mu\text{m}$ .

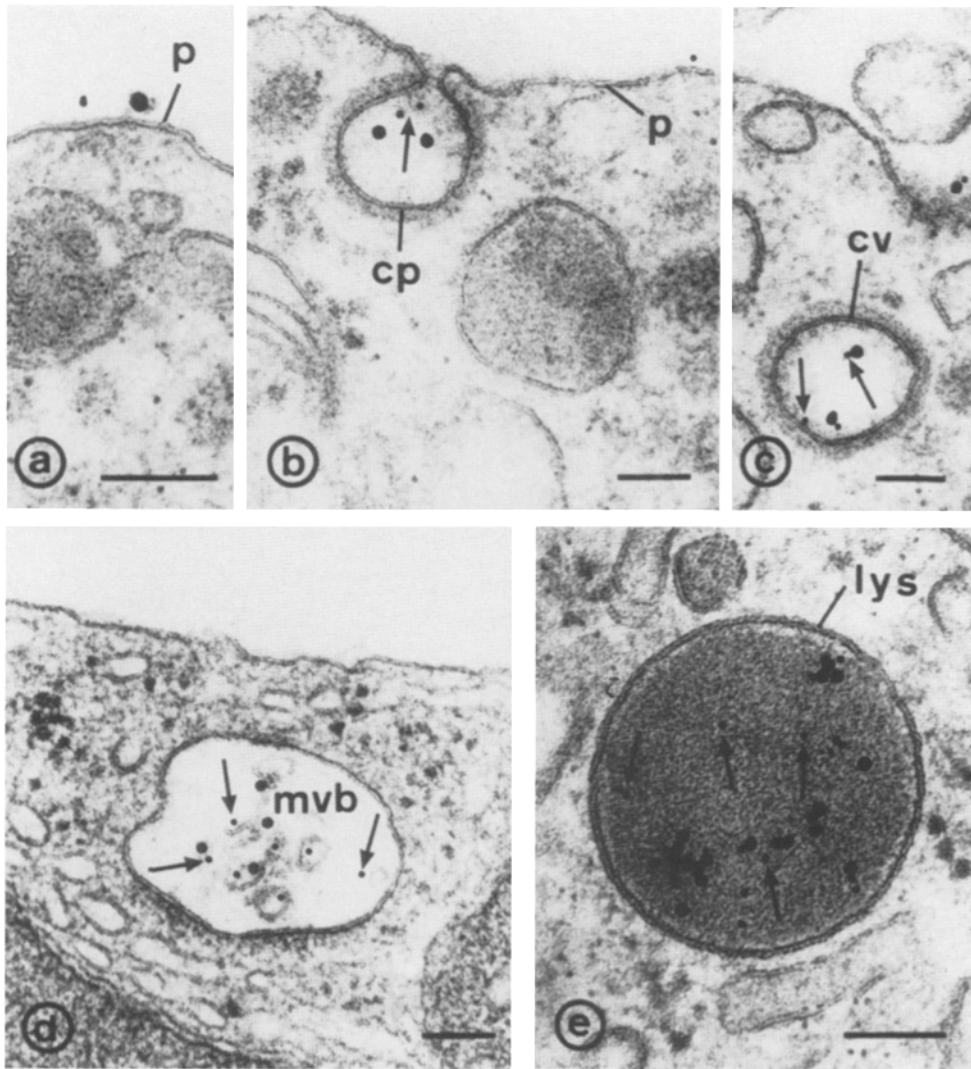
## Discussion

There have been several studies of the fate of hCG in target cells using either biochemical (5, 6, 14, 15, 45) or morphological (1, 2, 24, 28, 38) methods. However, no direct study of the LH receptor has been performed, at the electron microscopic level, probably due to the lack of specific antibodies. We have used here a monoclonal antibody directed against the porcine LH receptor which has many favorable features. It binds to the extracellular domain of the receptor but does not interfere with its functions (hormone binding, stimulation of adenylate cyclase) or with its rate of internalization. In addition, the antibody-receptor complex is very stable in an acidic environment similar to that of endosomes. The receptor was tagged with antibody-gold complex. This method has been used for EGF (12) and transferrin (16–19) receptors and has not been reported to yield artefactual results due to receptor interaction with the antibody gold particles.

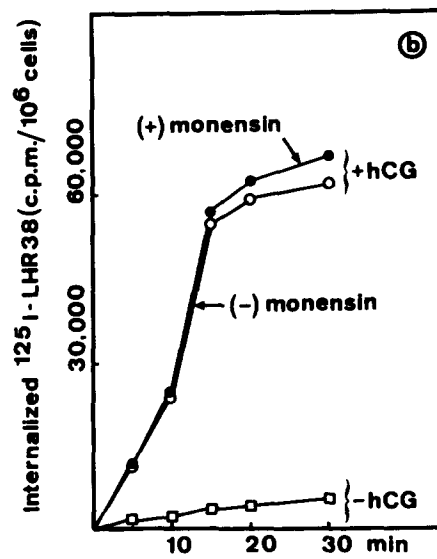
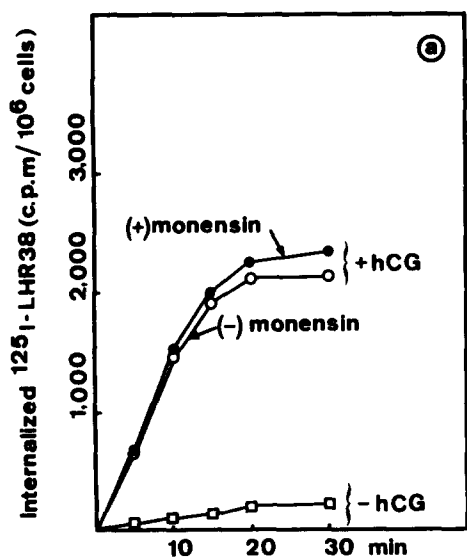
In the absence of hormone the receptor is distributed randomly on the cell surface, including in clathrin-coated pits. In this respect it is similar to the EGF receptor (11, 12) but is different from the  $\beta$ -adrenergic receptors (37). The presence of some molecules of the receptor in coated pits may explain the existence of the constitutive internalization

mechanism. The receptor molecules may be carried over by the formation of vesicles from the coated pits even in the absence of hormone. When the hormone is administered a higher proportion of receptors are clustered in the coated pits (with a concentration efficiency augmented 9.6-fold for Leydig cells and 8.6-fold for transfected L-cells) explaining the 11-fold increase in the internalization rate. Internalization signals have been determined in several receptors (for review and references see reference 42). In most cases they involve a tyrosine residue located in a tight turn environment (22). Such a structure is observed around tyrosine 632 of the pig LH receptor (25). This sequence is conserved in rat (30) and human LH (13) receptors. Mutagenesis studies will be necessary to determine the functional significance of this observation.

Raposo et al. (36, 37) have shown that both muscarinic acetylcholine receptors and  $\beta$ -adrenergic receptors are internalized through a smooth vesicle-associated pathway. A similar mechanism was observed by Payne et al. (34) studying the receptor for yeast mating factors which also belongs to the family of seven transmembrane receptors. Strosberg (41) discussed the possibility that it may be a general feature of the G-protein-linked receptors. He proposed that the latter being integral proteins well anchored to the membrane do not require the clathrin coat which is necessary for recep-



**Figure 7.** Leydig cells in culture: Double labeling with LHR38-Au<sub>5nm</sub> and hCG-Au<sub>15nm</sub>. The cells were incubated with the tracers for 30 min at 4°C, washed, and warmed at 37°C for 5 min (a-c), 15 min (d), and 60 min (e). Note that the markers label the same structures suggesting common internalization pathways for receptor (arrows) and hormone. cp, coated pit; cv, coated vesicle; lys, lysosome; mvb, multivesicular body. Bar, 0.1 μm.



**Figure 8.** Internalization and recycling of LH receptors in stimulated (+hCG) and control (-hCG) cells: effect of monensin. (a) Leydig cells; (b) transfected L-cells-expressing LH receptor. Cells were incubated for 30 min at 4°C with 0.67 μM [<sup>125</sup>I]LHR38 and then warmed at 37°C for various periods of time in presence or absence of 50 μM monensin, a drug which in other receptor systems (7, 8, 12, 16, 40, 47) inhibits both the synthesis and the return of internalized membrane receptors to the cell surface. The non-specific [<sup>125</sup>I]LHR8 binding was determined by incubating cells in the presence of unlabeled LHR38 (100 μg/ml). Note that (a) the addition of hormone augmented (~11-fold) receptor internalization, and (b) the internalization of [<sup>125</sup>I]LHR38 did not change significantly in cells receiving monensin.

tors with a single transmembrane domain. In contradiction to this hypothesis we show here that the LH receptor is internalized through the coated vesicle system. However, the LH receptor belongs to a peculiar subfamily of the G-protein-coupled receptors, having a long extracellular domain which binds the ligand. This feature may explain the difference between the LH receptors and  $\beta$ -adrenergic and muscarinic receptors. Electron immunocytochemical studies will have to be performed with other receptors belonging either to the subfamily of receptors binding ligand in their transmembrane domain or to the subfamily of receptors binding ligand in their extracellular domain. Only then a more general scheme could be proposed. Moreover, the fact that such similar receptors are internalized through different vesicle pathways opens the possibility of molecular studies of the involved mechanisms. It should be possible to construct chimeras between the LH receptor and for instance the  $\beta$ -adrenergic receptor to study their mechanism of internalization. Some such chimeras have already been obtained (32). This methodology should aid establishing which parts of the receptor molecule interact with elements (for instance the adaptor complexes; 9, 35, 43) of the various vesicles and how hormones modulate this interaction.

Previous studies based on hCG binding have led to divergent conclusions on LH receptor recycling to the surface: Ascoli (5, 6) suggested the absence of such a process, which on the contrary was observed by Genty et al. (14, 15). Our own observations rather support those of Ascoli: immunogold tagging of receptor and use of monensin both suggested a very limited extent of receptor recycling to the surface. Moreover, the fact that the hormone and the receptor share the same fate and are both targeted to the lysosomes also strengthens this conclusion. These results explain the important hormone-induced down-regulation of LH receptors (6, 45). In the case of the TSH receptor, physiological concentrations of the hormone provoke only a small decrease in surface receptor concentration (21). Now that mAbs have been produced against the latter receptor (26) it will be of interest to observe the differences in its cellular traffic compared to that of the LH receptor. The fact that LH receptor distribution and intracellular pathways are similar in the Leydig cells and in transfected L-cells is important for future studies. In vitro mutagenesis followed by introduction of expression vectors into easily transfectable L-cells will be used to analyze the domains of the receptor (and even individual amino acids) involved in its cellular traffic.

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