How Protein Hormones Reach Their Target Cells. Receptor-mediated Transcytosis of hCG through Endothelial Cells

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Abstract. In many organs the vascular endothelium forms a barrier which impedes the free diffusion of large molecules. The mechanism by which protein hormones are transported through the endothelial cells to reach their target cells is unknown. We have examined the transport of human chorionic gonadotropin (hCG) in rat testicular microvasculature by electron microscopy and by analysing the transfer of radiolabeled hormone and antibodies. Surprisingly, we have observed that the same receptor molecule which is present in target Leydig cells is also involved in transcytosis through the endothelial cells. The hormone was internalized by coated pits and vesicles on the luminal side of the endothelium. It was then localized in the endosomal compartment and subsequently appeared to be delivered by smooth vesicles into the subendothelial space. Moreover, anti-LH/hCG receptor antibodies were efficiently transported via the same system and delivered into the interstitial space. If generalized, these observations may define a new level of modulation of hormone action and may be of importance for drug targeting into the numerous organs which are responsive to the various protein hormones.

SYNTHESIS and secretion into the bloodstream of protein hormones have been extensively studied (review in Baulieu and Kelly, 1990). More recently experimental studies have focused on the interaction of the hormones with their target cells through specific receptors and on the ensuing activation of intracellular signalling pathways (review in Kahn, 1989). Surprisingly, almost nothing is known on the intermediary steps, i.e., the passage of hormones from the bloodstream into the interstitial space. Most capillary endothelial beds have occluding or tight intercellular junctions that do not permit the free diffusion of molecules of the size of protein hormones (review in Simionescu and Simionescu, 1991). It is thus necessary to hypothesize for these hormones the existence of as yet non identified transendothelial transport mechanisms. We have examined the passage of human chorionic gonadotropin (hCG)[1] through the continuous endothelium of the testicular microvasculature. We have observed that this transport occurs through a transcytosis mechanism involving the LH/hCG receptor. New regulatory mechanisms may thus exist at this level and may also have important consequences for the practicability of drug targeting.

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1. *Abbreviations used in this paper*: hCG, human choriogonadotropin; LH, luteinizing hormone; TSH, thyroid-stimulating hormone.

Materials and Methods

Animals

The experiments were carried out on male Wistar rats of about 200 g kept in standard housing and feeding conditions.

Chemicals

Human choriogonadotropin (hCG), iodination grade, was purchased from UCB-Bioproducts (Braine-l'Allend, Belgium), BSA (fraction V) and 1,2,4,6-tetrachloro-3α, 6α-diphenylglycouril were obtained from Sigma Immunochemicals (St. Louis, MO). Colloidal gold solutions were from Bio-Cell Research Laboratories (Cardiff, UK).

Antibodies

Mouse monoclonal anti-porcine luteinizing hormone (LH)/hCG receptor antibody 729 (LHR729) and mouse monoclonal anti-human thyroid-stimulating hormone (TSH) receptor antibody 51 (TSH51) were produced in mouse ascites and purified on protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) as previously described (Vu Hai et al., 1990; Loosfelt et al., 1992). The monoclonal antibody LHR729 raised against the pig LH/hCG receptor (Loosfelt et al., 1989) was chosen for its high cross-reactivity with rat hCG/LH receptor (Vu Hai et al., 1990). Mouse monoclonal anti-idiotypic antibody 10 (IDA 10) (Legrain et al., 1983) was produced from a hybridoma kindly supplied by Dr. P. Legrain (Unité de Génétique Somatique, Institut Pasteur, Paris). All monoclonal antibodies used in the present study were of the IgG1 isotype.

Tracers

Radioiodinated Proteins. [^{125}I]hCG (sp act 82.2 μCi/μg) was from NEN Research Products (Boston, MA). LHR729, TSHR51, and BSA were radio-

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iodinated by using 1,2,4,6-tetrachloro-3α,6α-diphenylglycouril as described (Fraker and Speck, 1978). Their specific radioactivity was >30 μCi/μg. The radioactive proteins were used at a final concentration of 0.1 μg/ml PBS, pH 7.3, containing 1 mg/ml of ovalbumin.

Gold Conjugates. LHR729-$Au_{5\,nm}$, TSHR51-$Au_{5\,nm}$, hCG-$Au_{5\,nm}$, hCG-$Au_{10\,nm}$, hCG-$Au_{15\,nm}$, and BSA-$Au_{10\,nm}$ complexes were prepared according to standard methods (DeMey, 1986). For perfusion experiments the gold complexes were resuspended in PBS, pH 7.3, containing 1 mg/ml ovalbumin to a final concentration corresponding to $A^{1\,cm}_{540\,nm} = 0.1$ for gold particles of 5 nm in diameter, to $A^{1\,cm}_{540\,nm} = 0.2$ for gold particles of 10 nm in diameter and to $A^{1\,cm}_{540\,nm} = 0.3$ for gold particles of 15 nm in diameter. In some experiments with BSA-$Au_{10\,nm}$ the concentration of the tracer was $A^{1\,cm}_{540\,nm} = 2$. The gold conjugates appeared as monodisperse when examined by electron microscopy. As previously described (Salesse et al., 1989; Ghinea et al., 1992) both hormone and anti-receptor antibodies coupled to colloidal gold preserve their biological activities. The gold-antibody complexes do not sterically hinder the binding of the gold-hormone conjugates and vice versa (Ghinea et al., 1992).

Perfusion Protocols. The animals were anesthesized with an intraperitoneal injection of 5% Nesdonal (Specia, Paris) (0.25 ml/100 g body weight). After thoracotomy and exposure of the heart the perfusion was carried out in an open circuit using the left ventricle as the inlet and the opened right atrium as the outlet. The vasculature was washed free of blood by perfusion with ~20 ml of prewarmed (37°C) PBS, pH 7.3, supplemented with 14 mM glucose and gassed with 95% O_2 and 5% CO_2. The perfusate was given under a constant pressure of 75 cm H_2O at a flow rate of 5 ml/min for 10 min. The tracers were then added to the perfusate and maintained in the circulation for various periods of time (5, 10, 15, and 20 min). The excess of unbound markers was washed out by perfusion with ~50 ml cold (4°C) PBS, pH 7.3 (in order to stop the transport process), in the same conditions as above. In some experiments the testes were removed, homogenized for counting of radioactivity and for immunoaffinity chromatography. In other experiments the testes were fixed in situ for 15 min and then overnight with a mixture of 2.5% glutaraldehyde and 5% formaldehyde in 0.1 M HCl-sodium cacodylate buffer, pH 7.2. In several experiments fragments of lung, diaphragm, heart, and epidydimal fat were excised and fixed for 90 min in the same fixative.

Controls. The specificity of the labeling by [^{125}I]hCG and hCG-$Au_{10\,nm}$ was assayed by perfusing the tracers in the presence of an excess of unlabeled hormone (45 μg of hCG/ml PBS, pH 7.3, containing 1 mg/ml ovalbumin). The specificity of the immunolabeling of LH/hCG receptors was tested by perfusion with either LHR729-$Au_{5\,nm}$ in the presence of 0.1 mg/ml unlabeled LHR729 or with TSHR51-$Au_{5\,nm}$ (used as a non-related control monoclonal antibody–gold complex).

Tissue Homogenization and Receptor Affinity Chromatography. During its transport towards the Leydig cells, hCG can either bind to the endothelial or to the Leydig cell receptors or be free (i.e., localized in the interstitial fluid). To follow the fate of the hormone we have perfused in situ the rat vasculature (see above) with [^{125}I]hCG. After 20 min of perfusion (at 37° or 4°C) the intravascular [^{125}I]hCG was washed out with cold (4°C) PBS, pH 7.3, containing 1 mg/ml ovalbumin. The testes were homogenized at 4°C with PBS, pH 7.3, containing a cocktail of protease inhibitors (1 mM benzamidine, 1 mM phenylmethylsulfonylfluoride, 5 mM leupeptin, 1 μg/ml pepstatin, 40 μg/ml aprotinin, and 100 μg/ml bacitracin) (buffer A). All further steps were carried out at 4°C. The homogenate was cold (4°C) centrifuged for 60 min at 105,000 *g* and the supernatant (S_1) saved. The pellet was extracted overnight with 1% Triton X-100 in buffer A and centrifuged for 60 min at 105,000 *g*. The resultant 105,000 *g* supernatant (S_2) was presumed to contain the membrane bound hormone (i.e., [^{125}I]hCG-LH/hCG receptor complexes). The supernatants S_1 and S_2 were diluted with 2 volumes of buffer A and then applied separately onto an immunomatrix column (0.25 ml of LHR729-Affigel 10) (Vu Hai, 1990) at a flow rate of 2 ml/h. The gel was finally washed with 50 volumes of buffer A and counted for radioactivity. IDA 10-Affigel 10 (Legrain et al., 1983) was used as a non-receptor related immunomatrix control.

Effect of the Hormone on the Transcytosis of LHR729 through the Endothelium. The transendothelial transport of [^{125}I]LHR729, fluorescein-LHR729, and LHR729-$AU_{5\,nm}$ was studied by perfusion in situ in the presence or in the absence of 4.5 μg of hCG/ml of PBS containing 1 mg/ml ovalbumin. TSHR51 (Loosfelt et al., 1992) was used as a control non-related monoclonal antibody.

Immunofluorescence Microscopy. After in situ perfusion with fluorescein-LHR729 (prepared according to Melan and Sluder, 1992) the testicular vasculature was fixed by perfusion for 30 min and then overnight with 3.5% formaldehyde in HCl-sodium-cacodylate, pH 7.2, buffer containing 10% sucrose. Cryostat sections (5–7 μm) were prepared from fixed rat testes and were collected on glass slides. The sections were examined in a Leitz orthoplan microscope using epi-illumination. Color and black and white photographs were taken with Kodak T-Max 400. Fluorescein-TSH51 (prepared according to Melan and Sluder, 1992) was used as a control non-related monoclonal antibody.

Electron Microscopy. Specimens collected from the fixed testes were cut into ~1 mm^3 blocks, and postfixed at 4°C for 90 min in 1% OsO_4, 0.1 M HCl-sodium cacodylate, pH 7.2, buffer. The blocks were stained with 0.5% uranyl acetate, dehydrated in graded ethanol and embedded in Epon 812. Sections (~60 nm), obtained with a Reichert OmUc ultramicrotome were stained with uranyl acetate and lead citrate and examined at 80 kV in a Siemens Elmiscop 101 microscope.

Capillaries, arterioles, and venules were identified as previously described (Simionescu and Simionescu, 1984).

Quantitation. A triplicate experiment was carried out to examine the changes provoked by hormone administration on the distribution and on the transcytosis of LHR729-$Au_{5\,nm}$. Ultrathin sections were cut from three randomly selected blocks of each experiment and 45 capillary profiles (120 endothelial cells) were examined. A total area of 1,500 μm^2 of endothelial cells was examined. Gold particles present on random cell profiles were allocated to one of the following organelles: luminal plasma membrane, coated pits associated with both luminal and abluminal endothelial fronts, coated vesicles, endosomes (smooth vesicles and tubular structures), multivesicular bodies, abluminal plasma membrane, and subendothelial space (endothelial basal lamina included). The percentage of gold particles associated with each organelle was determined. A total number of 750–800 gold particles were allocated to the different structures, in each experiment.

Results

Transcytosis Pathway of hCG in the Microvascular Endothelium of Rat Testis

To examine the route of the hormone through the vascular endothelium we coupled hCG to gold particles of various diameters, perfused rats at 37°C, and collected testes after various times of perfusion (5, 10, 15, and 20 min). We examined by electron microscopy the arteriolar, capillary, and venular endothelial cells. In all these cells and irrespective of particle size, the tracers were internalized and transcytosed by similar routes.

After 5 min of perfusion, the gold particles were distributed on the plasmalemma proper, the coated pits, and the coated vesicles of the luminal surface of the endothelium (Fig. 1 *a*). After 15 min of perfusion the labeled hormone was also present in the tubulovesicular endosomes (Fig. 1 *b*) and in large smooth vesicles located near the abluminal side of the endothelium (Fig. 1 *c*). At later stages (after 20 min of perfusion) the hormone–gold complex was located in large smooth vesicles open to the subendothelial space (Fig. 1 *e*). A large amount of free gold-labeled hCG was observed in the abluminal space and some tracer was apparently bound to Leydig cell membranes (not illustrated). Some particles were seen in the abluminal coated pits corresponding probably to the re-internalized hormone (Fig. 1 *f*). Moreover, a small proportion of the hCG-Au conjugate was present in the multivesicular bodies (Fig. 1, *d* and *f*) suggesting an accessory pathway of degradation of the hormone. In these highly pleomorphic structures the gold particles were concentrated on the limiting membrane and their tubular extensions (Fig. 1 *d*). In most cases, the internal vesicles of these vacuoles were devoid of labeling (Fig. 1, *d* and *e*). No transport of hCG-gold complexes was observed via plasmalemmal vesicles or through the junctions (Fig. 1 *a*) of endothelial cells.

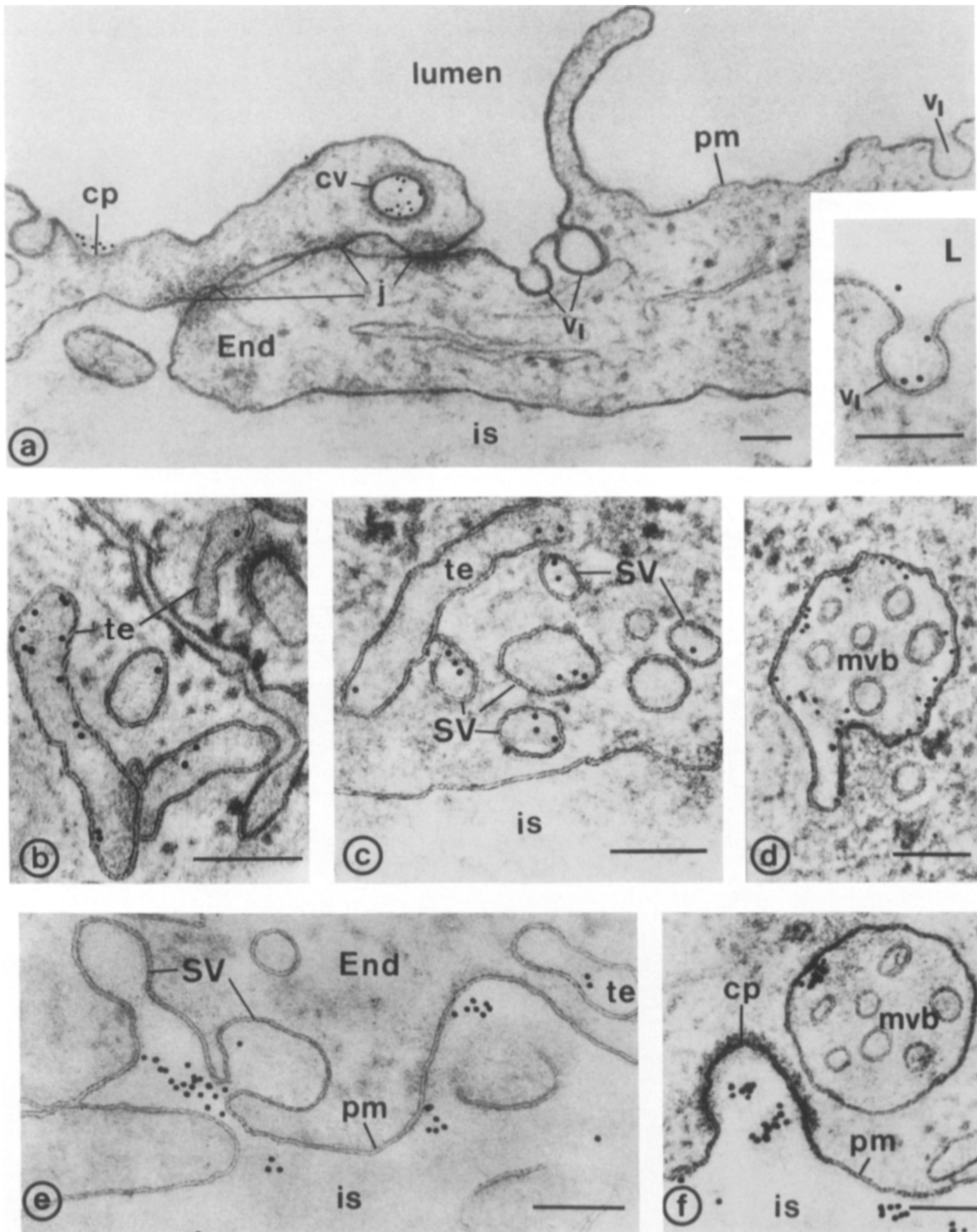
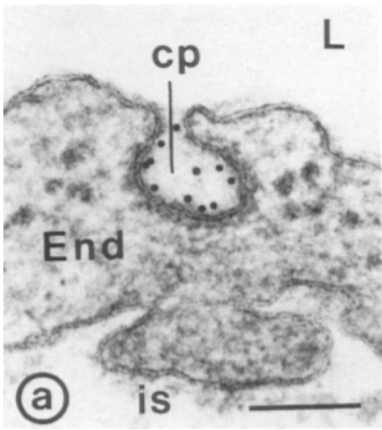


Figure 1. Transcytosis of hCG-Au$_{5\,nm}$ through the endothelial cells of rat testicular capillaries. (*a*) After 5 min of perfusion with hCG-Au$_{5\,nm}$ the tracer marks the luminal plasma membrane (*pm*), the coated pits (*cp*), and the coated vesicles (*cv*), but fails to label the plasmalemmal vesicles (v_l) open to the capillary lumen and the endothelial junctions (*j*). (*b–d*) After 15 min of perfusion the gold-labeled hormone is detected in the tubular (*te*) and vesicular (*ve*) endosomes (*b*) and in large smooth vesicles (*SV*) located near the abluminal side of the endothelium (*c*). Some hormone is present in the multivesicular bodies (*mvb*) (*d*). (*e* and *f*) After 20 min of perfusion hCG-Au$_{5\,nm}$ is delivered into the interstitial space (*is*) via smooth vesicles (*SV*) (*e*). Some of the tracer is associated with the plasma membrane (*pm*) and the coated pits (*cp*) of the abluminal cell surface of the endothelium (*f*). (*Inset*) Control perfusion with BSA-Au$_{5\,nm}$. The gold-labeled albumin was perfused for 5 min at a concentration 20-fold higher than the concentration of hCG-Au$_{5\,nm}$ ($A^{1\,cm}_{540\,nm}$ = 2 and $A^{1\,cm}_{540\,nm}$ = 0.1, respectively). Some gold particles are present in the plasmalemmal vesicles (v_l) open to the capillary lumen (*L*). *End*, endothelial cell. Bar, 0.1 μm.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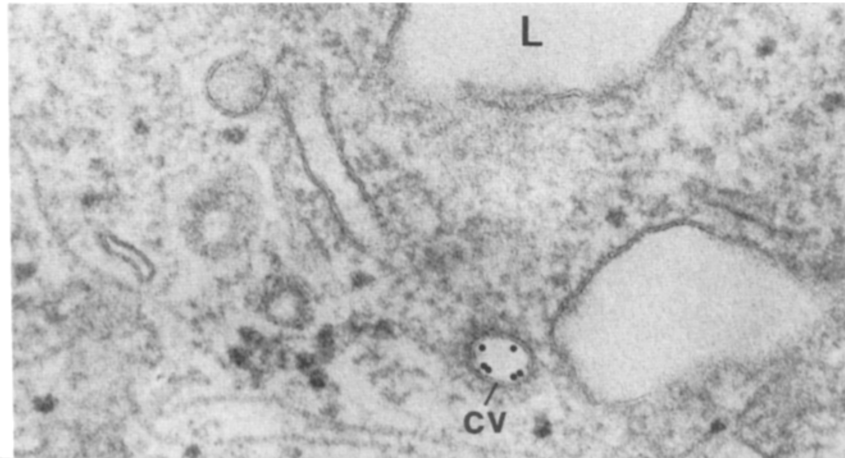
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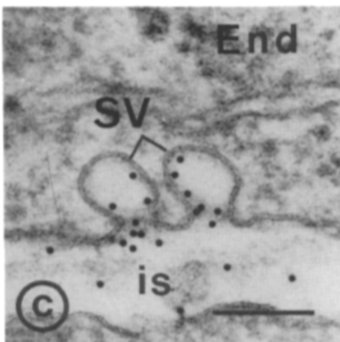
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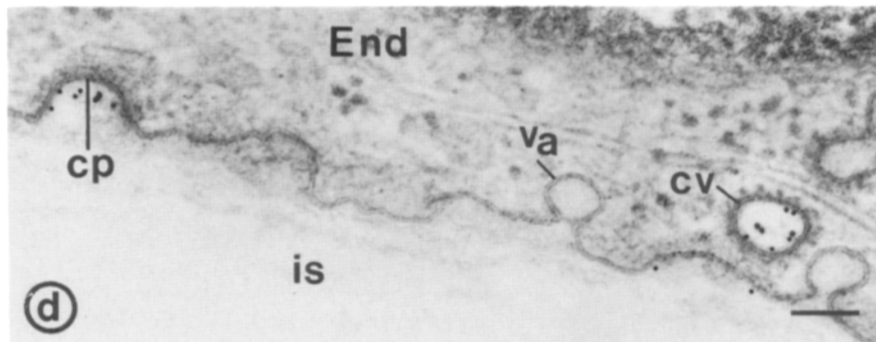
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As a control we used BSA coupled to colloidal gold particles. This tracer has been shown in the rat to cross via plasmalemmal vesicles the continuous endothelium of heart, lung, and adipose tissue (review in Simionescu and Simionescu, 1991). When the BSA-gold conjugate was perfused through the testes at a concentration similar to that of the hCG-gold complex, only rare particles were seen on the plasmalemma proper and in the plasmalemmal vesicles open to the luminal side of the endothelium (data not shown). At a 20-fold higher concentration of BSA-gold particles some of them were taken up by the plasmalemmal vesicles (never exceeding four particles per vesicle) (Fig. 1 *a*, *inset*) suggesting a bulk phase uptake. No BSA was seen passing through the endothelial junctions.

The LH/hCG Receptor Is Involved in Hormone Transcytosis

Observation of hCG-gold complexes in the endothelial cells of testes suggested that they interacted with a membrane protein which tended to accumulate in coated pits and coated vesicles. The similarity of this observation with the initial steps of hormone interaction with Leydig cells (Ghinea et al., 1992) led us to ask the question if the same receptor molecule could be involved in both cases.

We thus used a monoclonal antibody (LHR729) raised against the Leydig cell LH/hCG receptor and conjugated it to colloidal gold. Perfusion experiments were performed as above. After 5 min of perfusion at 37°C the antibody-gold complex was internalized by the endothelium. Internalization occurred via coated pits (Fig. 2 *a*) and coated vesicles in a way very similar to that previously observed for hormone-gold conjugate. After 15 min the probe was also observed in tubulo-vesicular endosomes and in large smooth vesicles located near the abluminal cell surface of the endothelium (Fig. 2 *b*). A small number of gold particles were seen in the subendothelial space. At longer times of perfusion (20 min) the proportion of the particles delivered into the subendothelial space via smooth vesicles open to the abluminal front increased as well as after hormone administration (Fig. 2 *c*, and Table I).

As in the case of hormone transcytosis, some antibody-gold complex labeled the abluminal coated pits and vesicles (Fig. 2 *d*) suggesting re-internalization of the antibody and thus, the recycling of the receptor.

Hormone and Receptor Molecules Have Similar Pathways of Transcytosis

To follow ultrastructurally on the same sections the cellular traffic of hormone and receptor we perfused in situ the testicular vasculature with a mixture of labeled hormone (hCG-$Au_{10\ nm}$) and of differently labeled anti-receptor antibody (LHR729-$Au_{5\ nm}$). The results of these double labeling experiments are shown in Fig. 3.

Table I. Effect of hCG on the Transendothelial Transport of LHR729-$Au_{5\ nm}$ in Rat Testicular Capillaries

Cellular organelles and subendothelial space	LHR729-$Au_{5\ nm}$ Particles	
	− hCG	+ hCG
Luminal plasmalemma	5.0	2.2
Luminal-coated pits	24.8	16.5
Coated vesicles	25.2	28.6
Endosomal structures (vesicles, tubules)	37.2	27.1
Multivesicular bodies	1.9	2.4
Abluminal plasmalemma	0.1	2.0
Abluminal-coated pits	0.2	4.9
Subendothelial space	5.6	16.2

The labeled antibody was perfused for 20 min as described in the absence or presence of hCG. The testes were examined by electron microscopy and the percentage of gold particles associated with each organelle was determined (see Materials and Methods). A total number of 2,400 and 2,250 particles were observed in the experiments in presence and in absence of hCG, respectively.

The hormone and the receptor molecules were colocalized on the luminal plasma membrane, in coated pits and vesicles, in tubulo-vesicular endosomes (Fig. 3, *a* and *b*), multivesicular bodies (Fig. 3 *c*) and smooth vesicles located near the abluminal front of the endothelium. After 20 min of perfusion both hormone and anti-receptor antibody were delivered into the subendothelial space (Fig. 3 *d*). Some particles of both tracers labeled the abluminal plasma membrane and its associated coated pits (Fig. 3 *d*).

These results suggested that the hormone was bound by the LH/hCG receptor on the luminal surface of endothelial cells, that the complexes were transcytosed and that the hormone was freed into the subendothelial space. Moreover, an antibody against the receptor could follow the same route and its transport through the endothelial cells could apparently be accelerated by administration of hormone.

Specificity of the Receptor-mediated Endothelial Transcytosis of hCG

To show that the transendothelial transfer of hCG was saturable we perfused the testes with hCG-gold complexes in the presence of a high excess of hCG (45 μg hCG/ml perfusate). This largely suppressed the binding of hCG-gold to the endothelial surface receptor and its subsequent transport (Fig. 4 *a*).

Tissue specificity was studied using both hCG and LHR729-gold particles. In organs having a continuous vascular endothelium (heart, lung, diaphragm, and epidydimal fat) both types of complexes failed to label the luminal plas-

Figure 2. Transcytosis of anti-LH/hCG receptor monoclonal antibody 729-gold complex in the capillary endothelium of rat testis. (*a*) After 5 min of perfusion LHR729-$Au_{5\ nm}$ the tracer is internalized by the endothelium (*End*) via the coated pits (*cp*) open to the capillary lumen (*L*). (*b*) After 15 min of perfusion, the tracer particles are observed in the coated vesicles (*cv*), tubular (*te*), and vesicular (*ve*) endosomes, and in large smooth vesicles (*SV*) located near the abluminal front of the endothelium (*End*). A small number of gold particles (*arrows*) are present in the interstitial space (*is*). (*c* and *d*) After 20 min of perfusion in the presence of 4.5 μg of hCG/ml the antibody-gold complex is delivered into the interstitial space (*is*) via smooth vesicles (*SV*) open to the abluminal cell surface of the endothelium (*End*) (*c*). Some tracer particles are associated with the abluminal coated pits (*cp*) and coated vesicles (*cv*) (*d*). v_a, abluminal plasmalemmal vesicle. Bar, 0.1 μm.

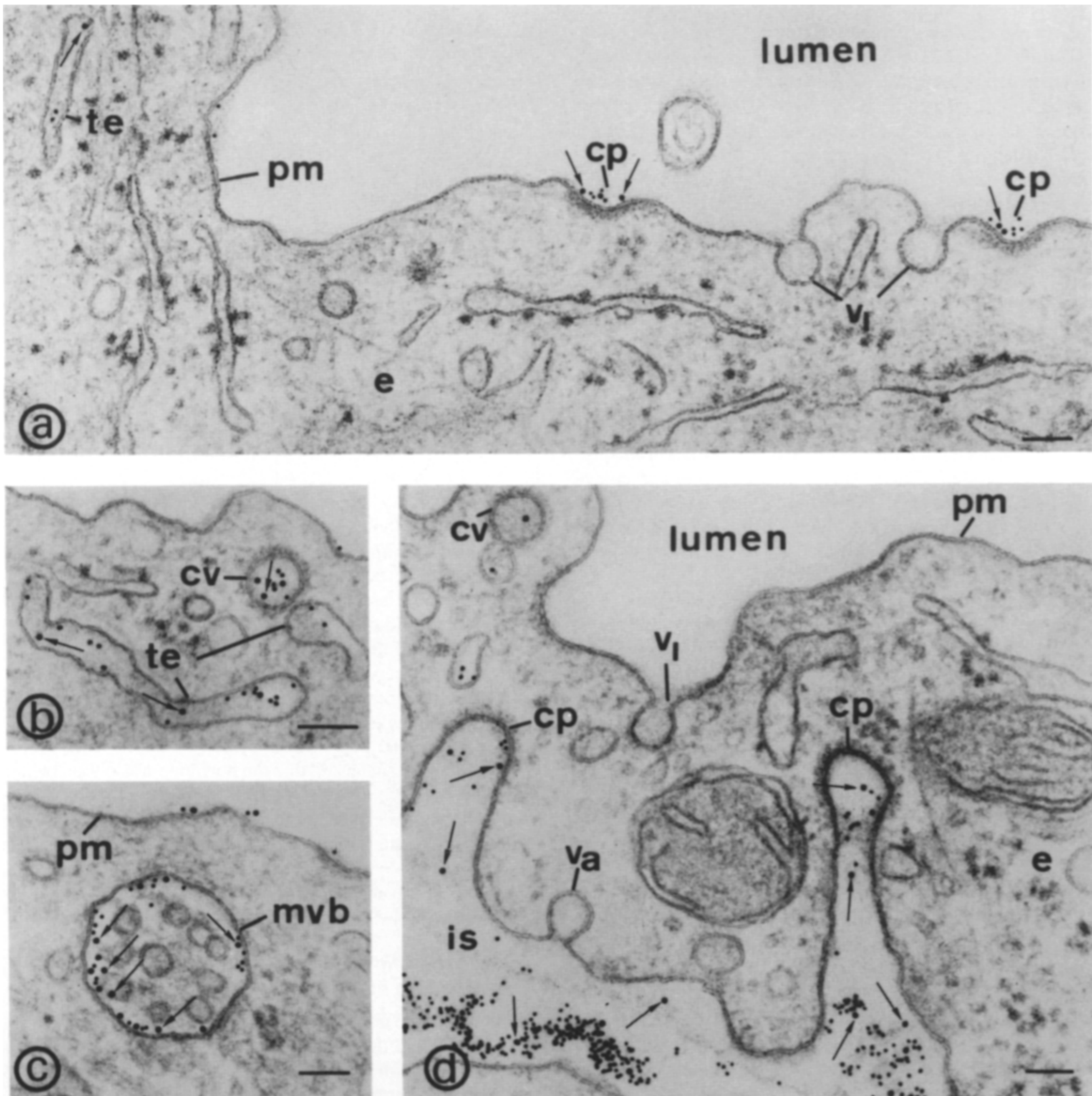

Figure 3. Comparison of the pathways of hormone and monoclonal anti-receptor antibody in testicular capillary endothelium. Double labeling with hCG-$Au_{10\ nm}$ and LHR729-$Au_{5\ nm}$. The testes were perfused in situ with a mixture of both tracers for 5 min (*a*), 15 min (*b* and *c*), and 20 min (*d*) at 37°C. Note that both markers label the same structures suggesting common transcytosis pathways for receptor and hormone (*arrows*). Note also the increased transfer of the antibody in the presence of hormone (compare with Fig. 2). *cp*, coated pit; *cv*, coated vesicle; *e*, endothelium; *is*, interstitial space; *j*, endothelial junction; *mvb*, multivesicular body; *pm*, plasma membrane; *te*, tubular endosome; v_a, abluminal plasmalemmal vesicle; v_l, luminal plasmalemmal vesicle. Bar, 0.1 μm.

malemma proper and its associated coated pits. Rarely, a very limited non-specific (fluid-phase) uptake of the tracers was observed occurring through plasmalemmal vesicles (at most one to two particles/vesicle) (Fig. 4, *b* and *c*).

Antibody specificity was studied by perfusing the testes with a gold-tagged monoclonal anti-TSH receptor antibody. In this experiment, some rare gold particles were observed in the plasmalemmal vesicles of endothelial cells (Fig. 4 *d*). Species specificity was analyzed by perfusing gold-tagged anti-LH/hCG receptor antibodies in pigs (LHR38 antibody) and in rabbits (LHR285 antibody). In both cases the labeling patterns of the endothelial cells in the testes were similar to those described above in rats (Ghinea, N., M. T. Groyer-Picard, and E. Milgrom, manuscript in preparation).

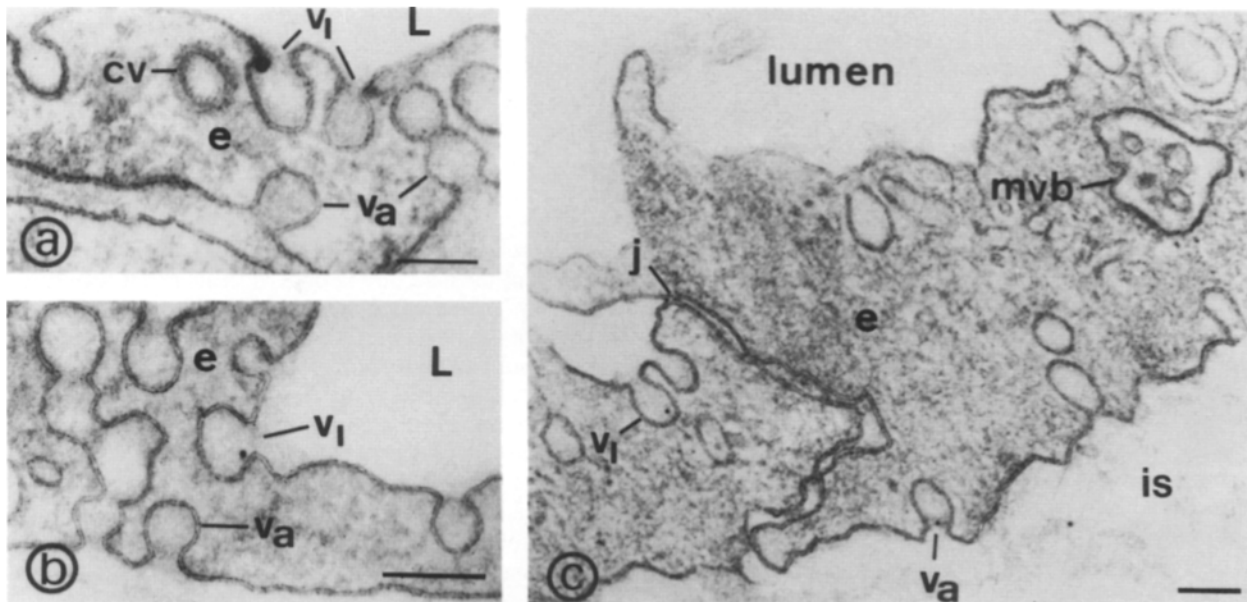

Figure 4. Specificity of the transcytosis of hCG and of anti-LH/hCG receptor antibody. (*a* and *b*) Tissue specificity was studied by analyzing the distribution of hCG-$Au_{15\,nm}$ (*a*) and of LHR729-$Au_{5\,nm}$ (*b*) in the microvasculature of the heart (myocardium). The tracers were perfused in situ for 20 min at 37°C. Note the very limited non-specific uptake of both tracers occurring via luminal plasmalemmal vesicles (v_l). Similar observations were recorded in other organs having a continuous capillary endothelium (lung, diaphragm, epidydimal fat). (*c*) The specificity of the transcytosis of antibodies was studied by perfusing the rat testes for 20 min at 37°C with mouse anti-human TSH receptor monoclonal antibody (TSH51) coupled to colloidal gold. Note that the TSH-$Au_{5\,nm}$ is transcytosed in fluid-phase via the plasmalemmal vesicles. *j*, endothelial junction; *L*, lumen; v_a, abluminal plasmalemmal vesicle. Bar, 0.1 μm.

Transendothelial Transport of ^{125}I-labeled hCG and Anti-receptor Antibody

Electron microscopic observations have allowed a detailed description of the cellular pathways which are followed for the hormone and the anti-receptor antibody. However, this method could not yield quantitative data and could not assess the possibility that the hormone delivered to the subendothelial space might be complexed to the extracellular domain of the receptor. To answer these questions we perfused the testes for 20 min with ^{125}I-labeled hormone. After extensive washing, we measured the free and the membrane-bound Triton X-100 extractable radioactivity. The latter represented the receptor bound hormone since it was displaced by coperfusion with an excess of unlabeled hormone and since 93% of it was retained by chromatography through an immunomatrix containing LHR729 antibody (Table II). The Triton-extractable radioactivity obtained after perfusion at low temperature most likely represents the hormone bound to the endothelial receptor. At 37°C transendothelial transport had occurred and there was in addition some binding to the Leydig cell receptors (Table II).

The soluble [^{125}I]hCG present in the testes probably represented the hormone which had been transported through the endothelium. This is supported by electron microscopy analysis which shows that unbound hCG–gold complexes are present in the subendothelial space (see Figs. 1 and 3). There was very little contamination by residual radioactive hormone remaining inside the blood vessels since soluble [^{125}I]hCG was largely suppressed (Table II) by coperfusion with unlabeled hormone or by perfusion at low temperature (blood contamination would not have been decreased by excess unlabeled hormone or by low temperature). Thus the soluble [^{125}I]hCG most likely represented a major fraction of the hormone which has passed the endothelial barrier (although some of this hormone has become attached to Leydig cell membranes) (see above).

Based on this assessment, the transport was negligible at 4°C. It was saturable, as shown by its suppression in the presence of an excess of unlabeled hormone (Table II). The solu-

Table II. Transendothelial Transport of [^{125}I]hCG in the Rat Testes

Experimental conditions	Membrane-bound hormone: Triton X-100 membrane extract (cpm/g wet tissue)	Membrane-bound hormone: Receptor bound	Soluble hormone: Total (cpm/g wet tissue)	Soluble hormone: Receptor bound
		%		%
37°C				
− hCG	2,700	93.2	2,200	1.6
+ hCG	400		360	
4°C				
− hCG	2,300		200	
+ hCG	350		130	

[^{125}I]hCG was perfused for 20 min at 4° or 37°C in the absence or in the presence of an excess of unlabeled hormone (see Materials and Methods). After washing by perfusion of PBS, testicular soluble, and membrane fractions were prepared. The latter was extracted by Triton X-100. Some of the extracts were chromatographed on an immunomatrix containing anti-LH/hCG receptor antibody (see Materials and Methods). The fraction of the hormone retained on the immunomatrix was considered as receptor bound. The Table shows one of the two experiments which yielded similar results.

*Table III. Transendothelial Transport of ¹²⁵I-Labeled Proteins: Anti-LH/hCG Receptor Antibody, Nonspecific (anti-TSH receptor) Antibody, and Albumin**

Tracer	Radioactivity (cpm/g wet tissue)	
	Soluble tracer	Triton X-100 membrane extract
− hCG		
[^{125}I]LHR729	2,600	12,850
[^{125}I]TSHR51	580	3,220
[^{125}I]albumin	930	5,900
+ hCG		
[^{125}I]LHR729	4,200	61,800
[^{125}I]TSHR51	700	3,740
[^{125}I]albumin	875	5,230

* Perfusion experiments were performed as described in Table II and in the Materials and Methods section.

ble [^{125}I]hCG might have been complexed with the ectodomain of the receptor. Cleavage of the poly-IgA receptor during the transepithelial transport of its ligand has been described (Mostov et al., 1980; Solari et al., 1989). To examine this hypothesis we perfused testes at 37°C, recovered the soluble fraction and applied it to an immunomatrix containing the LHR729 anti-receptor antibody (this antibody has been shown to recognize the extracellular domain of the receptor (Vu Hai et al., 1990)). Only 1.6% of the soluble [^{125}I]hCG did bind to the anti-receptor antibody suggesting that the hormone which has been transported through the endothelial barrier was not complexed to the receptor ectodomain.

Since electron microscopic experiments have suggested a transendothelial transfer of the anti-LH/hCG receptor antibody, we also labeled the latter with [^{125}I$^-$] and used it for perfusion experiments. For comparison we did similar perfusions with a non-specific antibody (^{125}I$^-$-labeled anti-TSH receptor antibody, TSHR51) and with [^{125}I$^-$]albumin. The experiments were performed both in the presence and in the absence of hCG.

As shown in Table III some of the anti-LH/hCG receptor antibody was recovered in the Triton X-100 membrane extract. This fraction could correspond to antibodies bound to the receptor. This fraction was increased ~60% by coperfusion of the antibody with hCG. This result may be explained by an increased transendothelial transport in the presence of the hormone (see also Figs. 2 and 3) leading to an increased binding of antibodies to Leydig cell receptors. No significant amounts of the albumin and of the non specific antibody were recovered in this Triton X-100 membrane extract (Table III). Coperfusion with hCG did not affect this fraction.

As discussed above in the case of [^{125}I$^-$]hCG, we considered the soluble [^{125}I$^-$]LHR729 recovered in the testes as representing a major fraction of the antibodies which had crossed the endothelial barrier. We thus observed that the transport of the anti-LH/hCG receptor antibody was markedly increased (approximately fivefold) by coperfusion with hCG. It was also 20-fold more efficient than that of the control non specific antibody and 12-fold more efficient than that of albumin.

To further examine the transport of the anti-LH/hCG receptor antibody and its subsequent binding to Leydig cell receptors we labeled this antibody with fluorescein, perfused it and examined by light microscopy the distribution of fluorescence in the testes (Fig. 5). As expected, the endothelial cells were strongly labeled (Fig. 5 *a*) and the antibody had reached the surface of the Leydig cells (Fig. 5 *b*). When a control fluorescein-labeled monoclonal antibody (Fluorescein-TSH51) was used only a weak signal was de-

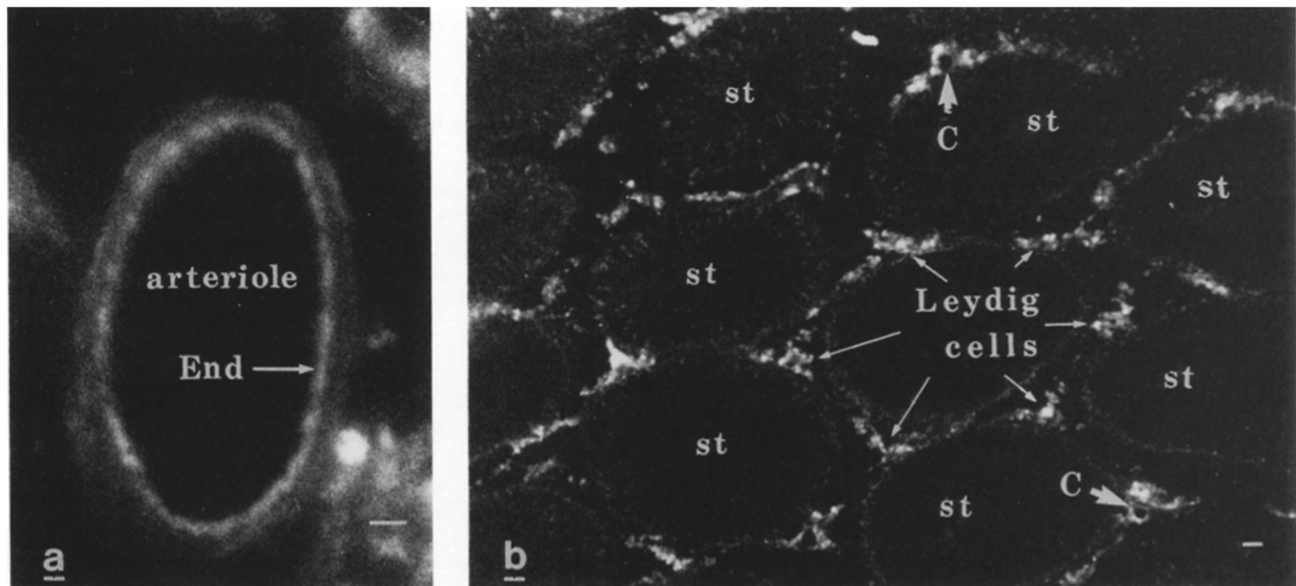

Figure 5. Intratesticular transfer of fluorescein-labeled anti-LH/hCG receptor antibody (LHR729). The tracer was perfused for 20 min at 37°C in the presence of 4.5 μg of hCG/ml. (*a*) Arteriolar endothelium (*End*) stained by the fluorescein-LHR729 conjugate. (*b*) The fluorescent probe is specifically associated with the Leydig cells and with the capillary endothelium (*c*). No fluorescence is detected at the level of the seminiferous tubules (*st*). Bar, 10 μm.

tected spread diffusely through the interstitial space (not shown).

Discussion

The microvascular endothelium forms a thin and continuous layer of squamous epithelial cells which separates the blood from the interstitial fluid. Endothelial cells are polarized and linked one to another by junctional complexes (tight and gap junctions) which ensure the continuity and mark the transition between the luminal (blood) and abluminal fronts. Ultrastructural and cytochemical studies (review in Simionescu and Simionescu, 1991) have shown that the microvascular endothelium constitutes a major barrier for the bidirectional exchanges of macromolecules between the plasma and the interstitial fluid. In vivo experiments involving injection of labeled proteins have confirmed this morphological finding showing that several hours are required for vascular/interstitial equilibration of radioactive plasma proteins (Larson et al., 1984; Powers and Bell, 1990; Ghitescu and Bendayan, 1992).

All these studies involved proteins present in relatively high concentration in blood. The route of the transendothelial transport of these proteins has also been studied. It was shown that they reach the interstitial space via plasmalemmal vesicles and transendothelial channels (Simionescu et al., 1975). This process involves a non-specific fluid phase mechanism (Williams, 1983) the efficiency of which is limited and depends on the number of plasmalemmal vesicles, on the plasma concentration of the proteins and on their size, shape, and charge (Simionescu et al., 1972; Ghinea and Simionescu, 1985; Ghinea and Hasu, 1986; Ghinea, 1987; Simionescu and Ghinea, 1990). It has also been observed that plasma proteins (for instance albumin) may interact in the plasmalemmal vesicles with low-affinity-binding proteins (Ghitescu et al., 1986; Milici et al., 1987; Ghinea et al., 1988; Schnitzer et al., 1988; Ghinea et al., 1989). It seems unlikely that protein hormones, which are present in blood at very low concentrations and which fluctuate rapidly in order to give sharp biological effects, could follow this route. Surprisingly very little information is available on the transendothelial transport of protein and glycoprotein hormones.

In the present study we have used both biochemical and morphological approaches to determine the transendothelial route of hCG towards its target Leydig cells in rat testis. We have observed its interaction with an endothelial cell membrane component which we have identified by immunocytochemistry and immunopurification (see Table II) as the LH/hCG receptor. Its interaction, in different species, with several monoclonal anti-receptor antibodies (LHR729, 38, and 285) recognizing different epitopes (V. Hai, unpublished results), confirmed this identification. Furthermore, a recent study confirmed the presence of LH/hCG receptor in rat ovarian and testicular blood vessels (Bukovsky et al., 1993).

The transcytosis of hCG occurs in several steps: the hormone molecules first bind to specific receptors expressed on the luminal front, then they are concentrated in coated pits and transferred via coated vesicles to an extensive smooth tubulo-vesicular endosome system (at present, the exact steps in the transition from coated vesicles to endosomes are not well characterized). The hormone and the receptor are then directed to the abluminal front by means of smooth transcytotic vesicles that pinch off from the tubular extensions of the endosomes. These vesicles became inserted into the abluminal endothelial plasma membrane and, finally, the hormone is released into the subendothelial space.

Our results also indicate that a small proportion of the hCG–gold conjugate leaves the endosomes and enters the multivesicular bodies. This fraction of hormone may thus be engaged in a pathway leading to its degradation. However, there is a possibility that some of the tracer is carried towards the abluminal cell surface by smooth transcytotic vesicles, derived from the tubular extensions of the multivesicular bodies. Such a mechanism has been reported for the transferrin receptor in other cell types (Harding et al., 1983; Hopkins, 1983).

The dissociation mechanism of the hormone–receptor complex on the abluminal cell surface is unknown. Covalent posttranslational modifications of the receptor, e.g., phosphorylation (Hipkin et al., 1993) and possible differences between the intraluminal pH of the transcytotic vesicles (derived from endosomes) and the physiological pH of the interstitial fluid may induce reversible conformational changes in the LH/hCG receptor molecule. These structural modifications would allow continuous hormone delivery and shuttling of the receptor across the endothelial cells. The reinternalization of the receptor on the endothelial abluminal cell surface seems to occur via coated pits and vesicles.

The route followed by hormone–receptor complexes in endothelial cells differs from the endocytosis previously described in Leydig cells and in transfected L cells (Ghinea et al., 1992). In the latter the complexes are directed towards the lysosomes. The tubulo-vesicular endosomes seem to be the place where different sorting mechanisms orient the hormone–receptor complexes along different pathways. This suggests the existence in Leydig and in endothelial cells of different signals on the cytoplasmic domain of the receptor (for instance different phosphorylation patterns) or of interactions with different "sorting" proteins (Goud and McCaffrey, 1991; Bomsel and Mostov, 1992).

Transcytosis, involving tubulo-vesicular endosomes has never, to our knowledge, been described in endothelial cells. However, such a mechanism has been observed in various epithelial cells for the transcytosis of thyroglobulin (Herzog, 1983), epidermal growth factor (Maratos-Flier et al., 1987), nerve growth factor (Siminoski et al., 1986), transferrin (Seddiki et al., 1992), IgG (Rodewald and Kraehenbuhl, 1984; Parham, 1989), IgM, and IgA (Geuze et al., 1984; Hoppe et al., 1985; Mostov and Simister, 1985). In the latter case some of the sorting mechanisms discussed above have been elucidated: Casanova et al. (1990) have shown that if the serine 664 of the polymer IgA receptor is replaced by an alanine the receptor keeps its property of internalization but fails to be sorted efficiently out of the endocytic route. Phosphorylation of serine 664 is thus probably important for the correct orientation of the receptor towards its transcytotic route.

Although we have used polyvalent tracers (hCG–gold and LHR729–gold complexes) to observe the fate of hormone and antibody, the morphological conclusions were consistent with other experiments involving iodinated or fluorescein-labeled monomeric equivalents.

How general is the mechanism of receptor-mediated hor-

mone transcytosis in endothelial cells is difficult to determine at present. We have gathered some preliminary evidence that hCG is transported through a similar mechanism in the ovarian microvasculature (N. Ghinea, unpublished results). Moreover, receptors for insulin and insulin-like growth factor II have previously been detected in blood vessels and the possibility of their involvement in hormone transport has been discussed (King and Johnson, 1985; Duffy and Pardridge, 1987; Bar et al., 1990; Pillion and Meezan, 1990; Moser et al., 1992; Pardridge, 1992). However, no specific mechanisms have been described and no ultrastructural studies have been performed. For low-density lipoproteins and for transferrin, involvement of specific receptors in the transendothelial transport has been described (Vasile et al., 1983; Jefferies et al., 1984; Soda and Tavassoli, 1984; Hashida et al., 1986; Kishimoto and Tavassoli, 1987; Omoto et al., 1992). Taken together these observations suggest that the mechanism described in the present study may be of more general significance. Further studies will be necessary to establish if this transport mechanism may be regulated: for instance if there is a hormone induced down-regulation of the endothelial receptor which could decrease hormone transport and contribute to the desensitization of hormone action. It will be also important to establish whether the receptor-mediated transcytosis is restricted to the endothelium of target organs or whether it also exists in hormone producing organs. In the latter case the transport of hormones would occur in the opposite direction (from the abluminal endothelial front to the vascular lumen). Hormone transport may also display different characteristics in fenestrated endothelia though protein transport does not seem to occur through the fenestrae (Simionescu et al., 1981).

Our results have also shown that the anti-receptor antibodies can be actively transported through the testicular endothelial barrier by the LH/hCG receptor-mediated transcytosis system. Drug targeting using monoclonal antibodies is extensively studied (reviewed in Goldberg, 1993; Rubin, 1993). Very often such antibodies linked to drugs or to toxins prove very effective in vitro on model cell culture systems but fail to be active in vivo in whole animals or in patients. Various hypotheses have been discussed in such cases (reviewed in Jain, 1989) but very few studies have addressed the question of the accessibility of the tumor. Kemshead et al. (1990) have shown that only 0.001% of a monoclonal antibody gains access to the interstitial space. Dillman et al. (1981) injected anti-melanoma antibodies in patients and observed a very low concentration in the tumors, contrasting with persisting high concentrations in blood. Thus an efficient transfer through the endothelial barrier may be a prerequisite for the therapeutic activity of such antibody conjugates. Yoshikawa and Pardridge (1992) have used anti-transferrin receptor antibodies to enhance the activity of drug targeting into brain tumors. The use of specific endothelial transcytosis systems and the enhancement of the transport of the antibodies by coinjection of hormones may be of great therapeutic interest. In the present study we have shown an efficient intratesticular transfer of the anti-LH receptor antibody linked to fluorescein. This is of course a preliminary observation and further studies of various practical aspects (extensive analysis of tissue specificity of transcytosis, hepatic clearance of antibodies, etc.) will have to be performed.

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