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Absence of Exon 10 of the Human Luteinizing Hormone (LH) Receptor Impairs LH, But Not Human Chorionic Gonadotropin Action

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The LH receptor (LHR) mediates the actions of LH and human chorionic gonadotropin (hCG). *In vivo* data showed that deletion of exon 10 does not affect hCG action, whereas LH action is impaired. To investigate the role of exon 10 in LH/hCG action *in vitro* we created stable COS-7 cells expressing the LHR with (wt) or without (−ex10) exon 10. Binding experiments showed that the affinities of LH and hCG to the LHR wt and −ex10 were similar. Stimulation of wt with hCG or LH resulted in increased cAMP. cAMP production was significantly impaired in –ex10 stimulated with LH. This response was not altered by pertussis toxin, excluding that G_i becomes activated in LHR –ex10. In desensitization experiments, intracellular cAMP of LHR wt and –ex10 declined to approximately 30%. No difference in intracellular cAMP was detected between LHR wt or –ex10 after recovery and restimulation with hCG or LH. These experiments show that impaired cAMP production of LHR –ex10 stimulated with LH is not due to anomalous receptor coupling or desensitization. We conclude that although exon 10 of the LHR plays no role in ligand binding, it is important for receptor activation by LH by a mechanism probably involving extracellular conformational changes. (*J Clin Endocrinol Metab* 88: 2242–2249, 2003)

THE ENDOCRINE REGULATION of gonadal function in the human depends entirely on the concerted action of the two gonadotropins, LH and FSH. In the male LH stimulates testosterone production by Leydig cells, whereas FSH controls the function of Sertoli cells and, together with testosterone, the production of mature gametes (1–3). Both gonadotropins are produced in the pituitary gland and function through binding to specific receptors exclusively localized in the gonads (3). The LH receptor (LHR) and the FSH receptor are G protein-coupled receptors characterized by a very large extracellular domain necessary for the recognition and binding of the respective gonadotropin. The structures of both gonadotropins and their receptors are well known, and their respective genes have been cloned and well characterized in the human as well as in several other species (4–7).

In the primate the LHR mediates the actions of two hormones: pituitary LH and chorionic gonadotropin (CG) (8, 9). In the male the LHR is located exclusively on Leydig cells in the testis and is fundamental for the production of testosterone. Although in the mouse CG/LH signaling during embryonic development is not necessary for testicular development (10), in the primate male sexual differentiation requires maternally produced human CG (hCG) and fetal LH to trigger testosterone production by Leydig cells, as suggested by several clinical observations in patients with inactivating mutations of the LH β-subunit (11) or LHR (6). LH is required for virilization and onset of spermatogenesis in the adult (3). LH and hCG share the same α-subunit, but have different β-subunits. The β-subunit of hCG differs from that of LH by the presence of a 24-amino acid extension at its C terminus (12, 13), which is presently not known to have any particular role in receptor binding or activation. The C-terminal extension of the hCG β-subunit carries additional glycosylation sites that confer a longer half-life and biopotency *in vivo* to hCG compared with LH.

The LHR gene consists of 11 exons and 10 introns (9). Exon 10 comprises 81 bp coding for 27 amino acids in the hinge region of the extracellular domain with no leucine-rich repeats (14). According to present knowledge, exon 10 of the LHR is not crucial for signal transduction by hCG (15). However, recent data from our group have shown that exon 10 of the human LHR plays an important role in hormone selectivity, as we have identified a deletion of exon 10 in a hypogonadal patient with a normal male phenotype (14). This indicates that maternal hCG interacted with the deleted LHR in the embryonal phase decisive for male development, whereas LH action was impaired at puberty, and sexual maturation failed to take place. In the human, inactivating mutations of the LHR result in impairment of Leydig cell function during fetal development and adulthood with the clinical picture of feminization of genotypical males known as Leydig cell hypoplasia types I and II (3). The present work further investigates the effects of deletion of exon 10 of the LHR on binding and signal transduction of hCG and LH *in vitro*.

Materials and Methods

Generation of pTracer vectors containing the LHR with (wt) or withouth (−ex10) exon 10

Human LHR wt and −ex10 cDNA had been inserted between the *Eco*RI and *Bam*HI restriction sites of the pSG5 vector (Stratagene, La Jolla, CA), which is suitable only for transient transfection (15). For stable transfection we therefore chose the pTracer vector (Invitrogen, Leek, The Netherlands), which contains a cytomegalovirus promoter in front of multiple cloning site 2 and the green fluorescent protein reporter gene,

Abbreviations: CG, Chorionic gonadotropin; −ex10, without exon 10; FCS, fetal calf serum; hCG, human chorionic gonadotropin; IBMX, isobutylmethylxanthine; LHR, LH receptor; PBG, PBS solution, 1 mM glucose, and 1% BSA, pH 7.4; PTX, pertussis toxin; wt, with exon 10.

which is fused to the selectable marker zeocine. This vector systems allows the noninvasive identification of transfected cells by imaging green fluorescent protein expression by fluorescence microscopy. The pTracer vector DNA was digested with *Eco*RI and *Eco*RV to generate suitable cloning sites for LHR cDNAs. LHR cDNAs were removed from the pSG5 vector by *Bam*HI restriction, followed by a fill-up reaction using Klenow polymerase to generate blunt ends for the pTracer *Eco*RV site and a second restriction by *Eco*RI. Ligation of the two cDNAs into the vector was performed according to the manufacturer's recommendation. The integrity and orientation of the cloned receptor cDNA were confirmed by DNA sequencing.

COS-7 cell lines

COS-7 cells (Cell Lines Service, Heidelberg, Germany) were cultivated in 100-mm polystyrene tissue culture dishes (IWAKI Co., Funabashi, Japan) in DMEM (Life Technologies, Inc., Paisley, UK) supplemented with 10% fetal calf serum (FCS; PAA Laboratories GmbH, Colbe, Germany). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 C and were split when approximately 50–70% confluent. One day before transfection cells were seeded at a density of 1×10^5 cells/30-mm dish in DMEM/10% FCS. Before transfection medium was removed, and the cells were washed with DMEM and incubated for 30 min with 1 ml serum-free DMEM at 37 C. Medium was removed, and 1 ml transfection solution was added with serum-free DMEM containing 2 μg plasmid DNA/well and Lipofectamine (Invitrogen) at a concentration of 62.5 μl/liter for liposome-mediated DNA transfection. After 5 h of incubation, 1 ml DMEM supplemented with 20% FCS was added. After 24 h, the medium was changed to DMEM, 10% FCS, and 1% zeocine. During the next 10–20 d the surviving cells were singularized by limiting dilution to obtain single-cell colonies. Highly purified (>95% pure) urinary hCG (iodination grade; specific activity, >16,000 IU/mg, WHO First International Reference Preparation) and LH from human pituitary (iodination grade; specific activity, >5,000 IU/mg; WHO First International Reference Preparation) was purchased from Calbiochem (La Jolla, CA).

Membrane preparation

Membranes were prepared essentially as previously described (16). Cells were plated in 100-mm cell culture dishes. Stably transfected COS-7 cells collected from 20 cell culture dishes were pooled and used for each lot of membrane preparations. Culture medium was aspirated, cells were washed twice with 1× Hanks' Balanced Cell Line Solution (Life Technologies, Inc.) with 0.01% glucose. Each plate was then incubated for 1 min with 5 ml trypsin-EDTA (Life Technologies, Inc.), and the cells were rinsed with 10 ml ice-cold Hanks' Balanced Salt Solution/0.01% glucose. Suspension was centrifuged for 5 min at 1,400 rpm, and the pellet was resuspended in 2 ml homogenization buffer (50 mM Tris-HCl, pH 7.5, and 27% sucrose). The suspension was homogenized at 18,000 rpm on ice with an Ultra-Turrax T25 (IKA Laborbedarf, Gottingen, Germany) with five strokes of 5 sec each at 30-sec intervals. The homogenate was diluted 4-fold with ice-cold homogenization buffer and centrifuged for 5 min at $160 \times g$. Supernatant was collected and spun at $25{,}000 \times g$ for 30 min. The pellet was resuspended in 35 ml ice-cold resuspension buffer (50 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$) and homogenized again by 10 strokes with a tight-fitting pestle in a glass homogenizer. The suspension was centrifuged at $25{,}000 \times g$ for 30 min, and the pellet was resuspended in ice-cold resuspension buffer (1 ml/dish). The protein concentration of the membrane pool was determined by bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL), and aliquots of 250 μl were snap-frozen and kept at –80 C.

Binding experiments

Incubation of binding/displacement assays with membrane preparations, cell homogenates and whole cells was carried out in the presence of 25 mM Tris-HCl, 1 mM EDTA, and 1.25% BSA. After incubation, cells/membranes were washed with 1.5 ml ice-cold 2 mg/ml bovine γ-globulin in 0.1 M NaCl, followed by 0.5 M ice-cold 20% polyethylene glycol (Sigma-Aldrich Corp., Deisenhofen, Germany). Well vortexed mixtures were left on ice for 10 min and centrifuged for 10 min at 3500 rpm in a cryofuge, and supernatants were discarded. The precipitates were resuspended in 1.5 ml 0.1 M NaCl, with further addition of 0.5 ml 20% polyethylene glycol. Radioactivity was determined with a gamma counter (LKB 1277, Wallac, Inc., Turku, Finland).

Membrane preparations (15 μg/ml) were exposed to rising levels of [^{125}I]hCG in the range of 28–600 pM (labeled by Hartmann Analytic, Braunschweig, Germany). The specific activity of the [^{125}I]hCG was 480 kBq/μg (17.8 MBq/nM; purity, >95%). For whole cell binding experiments cells were seeded 1 d before incubation in 5-ml plastic tubes (Sarstedt, Numbrecht, Germany) at a density of 100,000 cells/tube. The incubation time of the cells with labeled hCG was 90 min at 37 C (n = 2) or overnight (12 h) at room temperature (n = 1) with the same results. The cells were exposed to [^{125}I]hCG concentrations in the range of 10–900 pM and washed with 1.5 ml ice-cold 2 mg/ml bovine γ-globulin in 0.1 M NaCl with 0.05% Triton X-100. For cell homogenate binding experiments cells were washed once with 1.5 ml PBG (PBS solution, 1 mM glucose, and 1% BSA, pH 7.4), and 250 μl serum-free DMEM with 42 μl/ml protease inhibitor cocktail (P8340, Sigma-Aldrich Corp.) were added. The cells were then homogenized by five strokes of an Ultra-Turrax of 5 sec each at 18,000 rpm and incubated.

Displacement experiments

Cell membrane preparations (10 μg protein/tube) were exposed to a fixed dose of 350 pM labeled hCG. Rising concentrations of unlabeled hCG/LH were added in the range of each 1 fM to 35 nM. After 90 min of incubation at 37 C the membranes were treated following the above-mentioned protocol, and radioactivity was determined.

Calibration of hormones

Highly purified LH and hCG hormone preparations (>95% pure; Calbiochem) were calibrated *in vitro* against the International Standard for LH WHO 80/522. COS-7 cells permanently expressing wt LHR were seeded in a 96-well plate at a density of 20,000 cells/well. The cells were incubated in the presence of 25 mM isobutylmethylxanthine (IBMX; Sigma-Aldrich Corp.) for 150 min in 250 μl PBG with increasing concentrations of hCG, LH, or WHO 80/522 in the range of 1333–0.16 IU/liter (conversion factor: 1 IU/liter = 5.815 pM). After incubation the wells were frozen at –20 C and thawed, and triplicate samples were pooled and boiled at 100 C for 2 min. Supernatants were stored for total cAMP measurement.

Signal transduction/cAMP measurements

Each cell line was seeded 1 d before the experiment in a 96-well plate at a density of 8000 cells/well. Cells were stimulated in triplicate for 180 min at 37 C with LH or hCG at a range of 0.076–40,000 IU/liter (in terms of WHO LH80/522) in 250 μl PBG. After incubation the wells were frozen at –20 C and thawed, and triplicate samples were pooled and boiled at 100 C for 2 min. cAMP production of the COS-7 cells was measured by ELISA (IHF, Hamburg, Germany). The pooled samples of triplicate stimulations were measured in duplicate. In experiments with low cAMP values, cAMP ELISA with acetylation (sensitivity, 0.04 nmol/liter) was used, whereas in experiments with IBMX, cAMP ELISA without acetylation (sensitivity, 1 nmol/liter) was used.

For the experiments using pertussis toxin (PTX) stably transfected with COS-7 cells, LHR wt and LHR –ex10 were seeded the day before the experiment in a 48-well plate at a density of 25,000 cells/well. Cells were incubated 24 h with medium or 100 μg/liter PTX (Sigma-Aldrich Corp.), DMEM, and 10% FCS. The medium was removed, and the cells were stimulated in the presence and absence of PTX with 0, 1000, and 4000 IU/liter LH or hCG for 60 min at 37 C. The medium was removed, and cells were washed once with 200 μl DMEM. Cells were immediately frozen and thawed after 1 h, and intracellular cAMP production and protein content were determined. In positive control experiments 10% FCS inhibited basal cAMP production, which was increased to baseline by the addition of 100 μg/l PTX (data not shown).

For desensitization experiments, LHR wt and LHR –ex10 cells were seeded the day before the experiment in a 48-well plate at a density of 25,000 cells/well. Cells were desensitized with 10^4 IU/liter hCG for 2 h, washed twice with 300 μl ice-cold DMEM, then incubated with 300 μl ice-cold PBS, 50 mM glycine, and 100 mM NaCl, pH 3, for 5 min at 4 C to remove the bound hormone (16, 17). Medium was removed, and 250

μl DMEM were added; cells were kept for recovery 0, 5, 10, 20, 40, 60, 90, and 120 min at 37 C before stimulation with 4000 IU/liter hCG or LH for 60 min. After stimulation, cells were washed once with DMEM and immediately frozen at –20 C. Intracellular cAMP and protein contents were determined as described before.

Statistical analysis

Binding analysis was performed using PRISM 3.0 (GraphPad Software, Inc., San Diego, CA). Statistical analysis was performed by *t* test (Excel 97, Microsoft Corp., Redmond, WA) and SigmaStat (SPSS, Inc., Chicago, IL). Results are presented as the mean ± SD.

Results

Of several clones expressing wt LHR or the –ex10 LHR we selected and further cultured those with similarly high cAMP production when stimulated with hCG. Binding saturation experiments with iodinated hCG and membrane preparations were carried out with the selected clones with or without exon 10. After Scatchard analysis, two cell lines with 44,500 receptors/cell (LHR wt) and 51,000 receptors/cell (LHR –ex10), respectively, were selected for further experiments. No statistically significant difference was observed in K_d values in binding experiments between the cell lines (LHR wt K_d = 814 ± 68 pM; LHR –exon 10 K_d = 882 ± 54 pM; n = 3; Fig. 1), showing that exon 10 does not influence the binding characteristics of [^{125}I]hCG.

To determine the percentage of internalized receptors and those expressed on the cell surface, we repeated saturation experiments with whole cells, cell homogenates, and membrane preparations with labeled hCG for 90 min at 37 C (n ≥ 3). Analysis of [^{125}I]hCG binding with independent cell preparations showed that of 44 ± 9% of the total binding sites are at the cell surface in the LHR wt cell line *vs.* 67 ± 19% of the total receptors in the LHR −ex10 cell line (Fig. 2). Therefore, in permanently transfected COS-7 cell lines selected on the basis of similar responses to hCG in terms of cAMP production, we did not observe the failure of receptor trafficking to the cell surface in the absence of exon 10, as previously reported (15).

To investigate the affinities of both LH and hCG to LHR wt and LHR –ex10, we carried out displacement experiments using [^{125}I]hCG and increasing doses of unlabeled LH/hCG. Displacement experiments using membrane preparations (n = 4) showed that binding of LH to wt or −ex10 LHR was not significantly different (IC_{50}: wt, 12.96 ± 5.83 pM; –ex10,

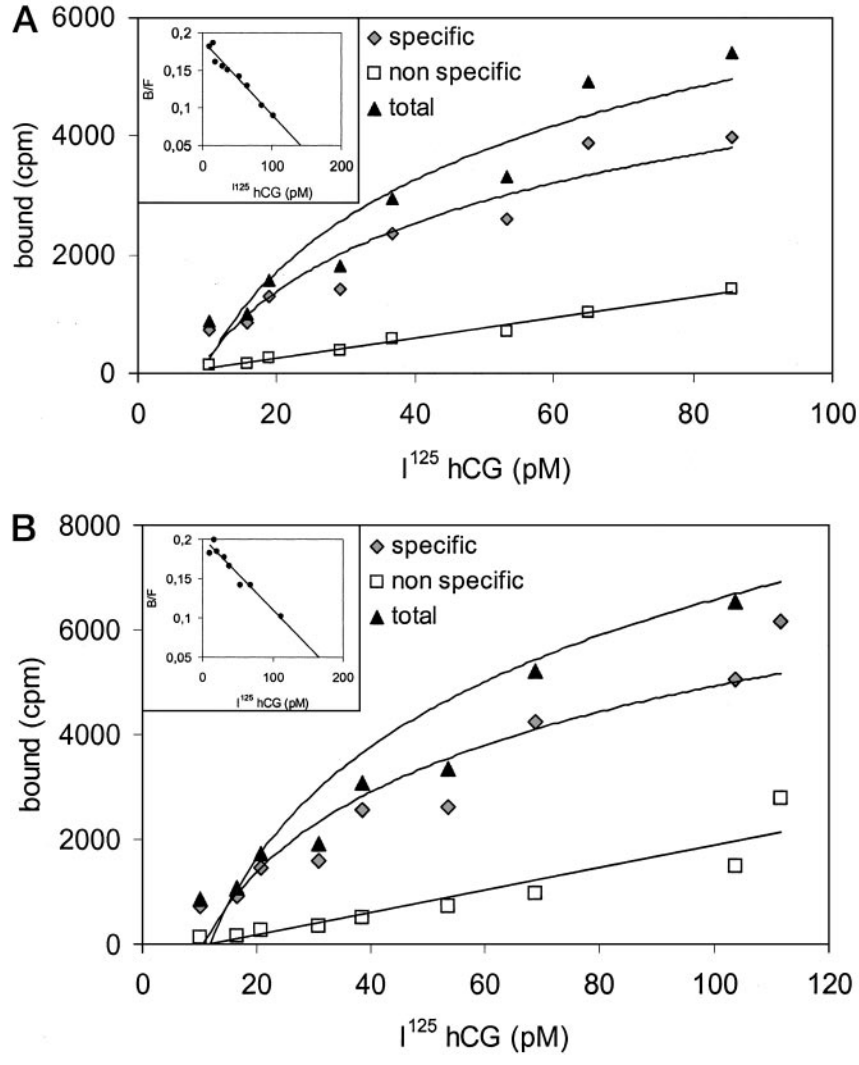

FIG. 1. Binding curve and Scatchard analysis of [^{125}I]hCG binding to membrane preparations of LHR wt (a) and LHR −ex10 (b). Analysis of three independent experiments showed no statistically significant difference in binding to LHR wt (K_d = 814 ± 68 pM) and LHR –ex10 (K_d = 882 ± 54 pM).

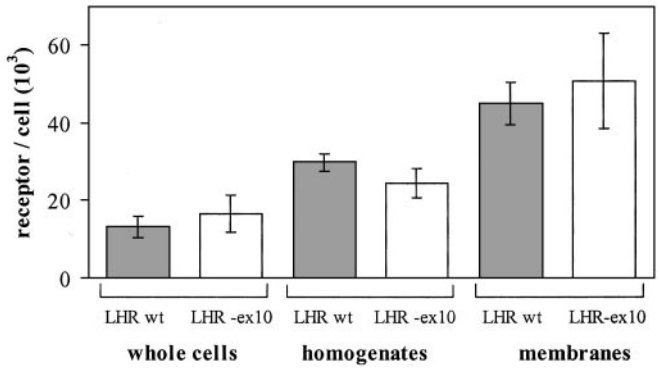

FIG. 2. Analysis of receptor numbers (mean ± SD) in COS-7 cell lines permanently expressing the LHR with or without exon 10 in whole cells, cell homogenates, and membrane preparations thereof (n ≥ 3). Some 44 ± 9% of receptors in LHR wt and 67 ± 19% of receptors in LHR −ex10 (whole cells *vs.* cell homogenates) were expressed at the cell surface. No statistically significant difference between the percentage of receptor traffic could be observed, especially no impairment in receptor expression of LHR −ex10 ($P > 0.05$, by t test).

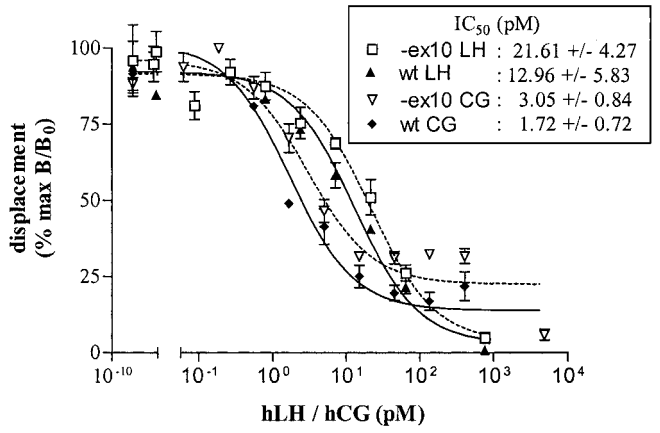

FIG. 3. Displacement of a fixed dose of [^{125}I]hCG by rising levels of unlabeled hCG/LH in the COS-7 cell line stably expressing the LHR wt or −ex10. Results indicate the mean ± SD of four independent experiments with membrane preparations incubated for 90 min at 37 C. Neither hCG nor LH showed a significant difference in IC_{50} values when incubated with LHR wt or LHR −ex10 receptors. However, there was a significant difference in IC_{50} values between the two gonadotropins independent of receptor type ($P < 0.05$, by t test).

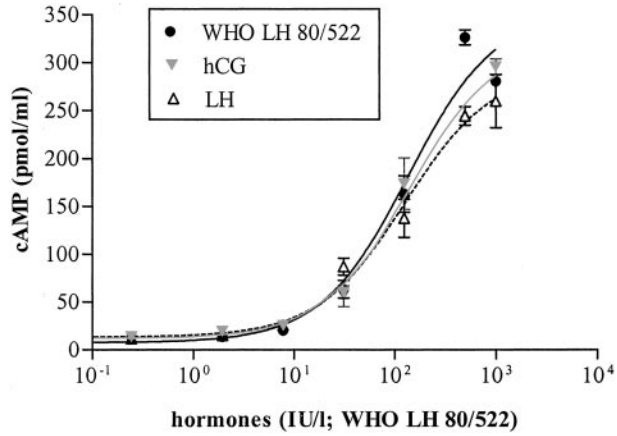

FIG. 4. Equipotency of highly purified preparations of hCG and LH on LHR wt cells calibrated against WHO standard LH 80/522 (conversion factor: 1 IU/liter = 5.815 pM). Some 20,000 cells/well were stimulated in presence of IBMX for 150 min with rising doses of each hormone, and total cAMP was measured. The figure shows one of five dose-response curves obtained after calibration. Calibration was repeated for each new lot of hormones used for the experiments.

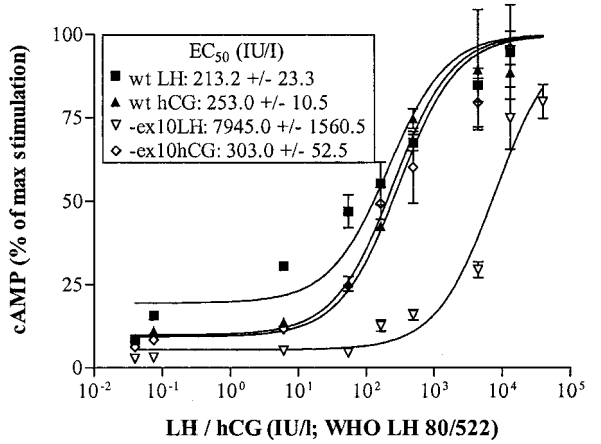

FIG. 5. Total cAMP production in cell line LHR wt or LHR −ex10 expressing approximately the same receptor number. Stimulation of cell lines was performed with graded doses of hCG and LH previously calibrated against WHO LH 80/522. Results presented are the mean ± SD of three independent experiments (conversion factor: 1 IU/liter = 5.815 pM).

21.61 ± 4.27 pM; n = 4; Fig. 3). However, LH had a significantly lower potency than hCG in displacing [^{125}I]hCG (IC_{50}: LH, 12.96 ± 5.83 pM; hCG, 1.72 ± 0.72 pM; $P < 0.05$; n = 4; Fig. 3). We conclude that exon 10 is not important for binding saturation of LH and hCG to the LHR.

In further experiments we analyzed whether LH and hCG are equally effective in inducing cAMP production in the presence and absence of exon 10. However, LH and hCG are very heterogeneous hormones due to their high carbohydrate content causing various molecular isoforms with different sugar compositions and molecular weights. Therefore, equimolar concentrations of hCG and LH may result in quite different bioactivities of the hormones *in vitro*. We therefore determined the specific activity of both hormone preparations in terms of WHO LH 80/522 *in vitro*, using LHR wt cells. Specific activity was 5,360 IU/mg for LH and 14,980 IU/mg for hCG in terms of WHO LH 80/522. As shown in Fig. 4, after calibration against WHO LH 80/522, hCG and LH were equipotent *in vitro* in the wt LHR. Calibration was repeated for each new lot of hormones, and equipotent doses of LH and hCG were used.

Total cAMP production measured in LHR wt cells stimulated with hCG or LH showed no significant difference in cAMP production (EC_{50}: hCG, 303.0 ± 52.5 IU/liter; LH, 213.2 ± 23.3 IU/liter; n = 3), but it was significantly impaired in −ex10 cells stimulated with LH compared with hCG (EC_{50}: LH, 7945.0 ± 1560.5 IU/liter; hCG, 253.0 ± 10.5 IU/liter; $P < 0.05$; n = 3). Although cAMP was produced by LH stimulation of LHR −ex10, approximately 30 times higher concentrations of LH were required to induce the cAMP production observed in the presence of LHR wt (Fig. 5). This finding explains why the mutated LHR previously described (14)

could react *in vivo* to exogenous hCG, but not to endogenous LH. Thus, exon 10 of the LHR is necessary for induction of full signal transduction by LH, but not by hCG.

The LHR has been reported to be coupled to G_i, leading to phospholipase C activation (17). To investigate whether the absence of exon 10 leads to anomalous coupling of G_i to the LHR, reducing cAMP production in the presence of LH stimulation, we carried out experiments with PTX to inhibit G_i. Stably transfected LHR wt and LHR –ex10 cells preincubated with PTX were stimulated with equipotent doses of LH and hCG (Fig. 6, a and b). The doses of hCG and LH were chosen to obtain a submaximal response in the case of LHR wt and LHR –ex10 when stimulated by hCG, but a negligible response in the case of LHR –ex10 stimulated by LH (Fig. 5). Intracellular cAMP determination showed an approximately 4.6-fold significant increase in cAMP after each stimulation with LH/hCG with or without PTX in LHR wt cells (by *t* test $P < 0.05$; Fig. 6a), with no significant difference in the absence or presence of PTX. LHR –ex10 cells showed the same significant increase in cAMP when stimulated by hCG with or without PTX, but no significant cAMP production over the basal level when stimulated with an equipotent dose of LH (Fig. 6b). Addition of PTX did not further increase cAMP production ($P > 0.05$). Therefore, PTX could neither influence hCG-stimulated cAMP production nor rescue the LH response in the absence of exon 10 of the LHR.

As another possible cause of the low cAMP formation in LHR –ex10 stimulated with LH, we considered increased receptor desensitization compared with LHR wt. In desensitization experiments preincubation of LHR wt and LHR –10 with 10^4 IU/liter hCG or LH showed a significant reduction of cAMP production in both cell lines (Fig. 7, a and b), as described by others (18–20). LHR wt cells desensitized by hCG showed a decline in cAMP production of 16% in the case of LH and 24% in the case of hCG (Fig. 7a). cAMP production by LHR –ex10 cells was not significantly different from basal levels after stimulation with LH and was reduced 29% after stimulation with hCG (Fig. 7a). Basically the same results were observed when cells were desensitized by LH. cAMP production declined to 16% in desensitized wt cells stimulated with LH and to 25% after hCG stimulation (Fig. 7b). In LHR –ex10 cells, cAMP levels were not significantly different from basal levels after LH stimulation, whereas cAMP production dropped 35% when desensitized cells were stimulated by hCG (Fig. 7b). No statistically significant difference in recovery of intracellular cAMP production could be detected between LHR wt or LHR –ex10 cells up to 120 min of recovery and restimulation (data not shown). Thus, we conclude that impaired cAMP production of LHR –ex10 cells when stimulated with LH is not due to increased desensitization of the receptor.

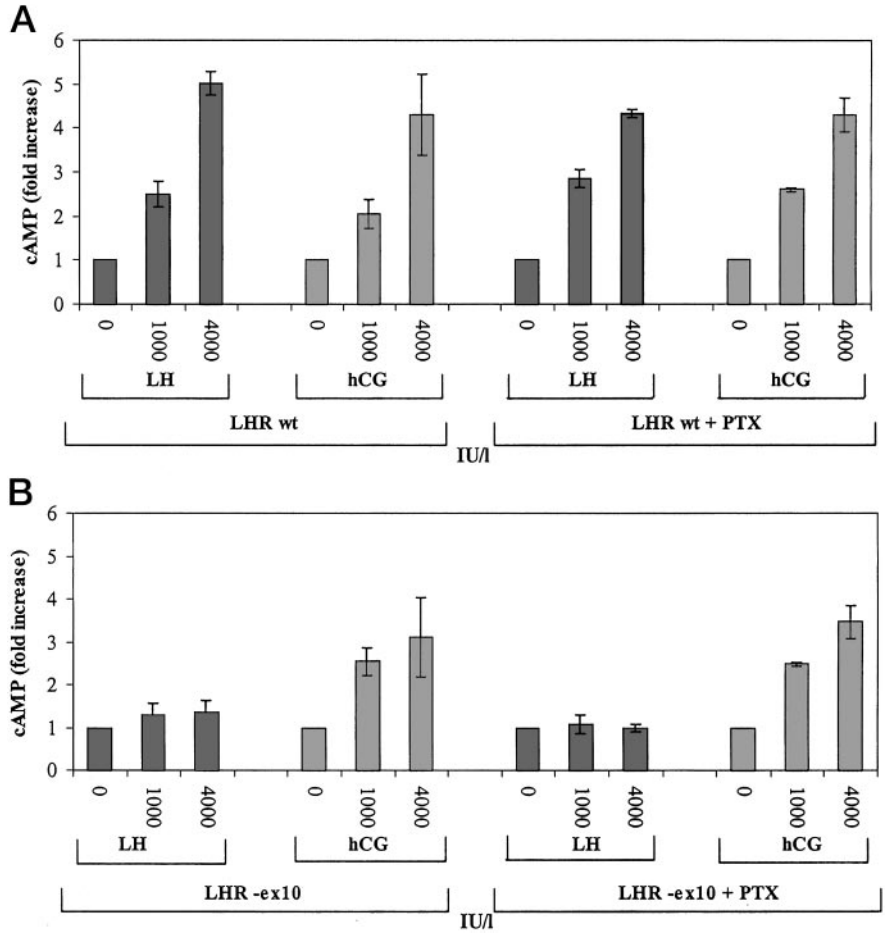

FIG. 6. Intracellular cAMP production in cell lines permanently expressing LHR wt (a) or LHR –ex 10 (b). Stimulation of whole cells was performed with previously calibrated hormone preparations of hCG and LH using 0, 1000, and 4000 IU/liter, respectively, in the presence and absence of PTX. Results are presented as the fold increase over the control value (mean ± SD of three independent experiments).

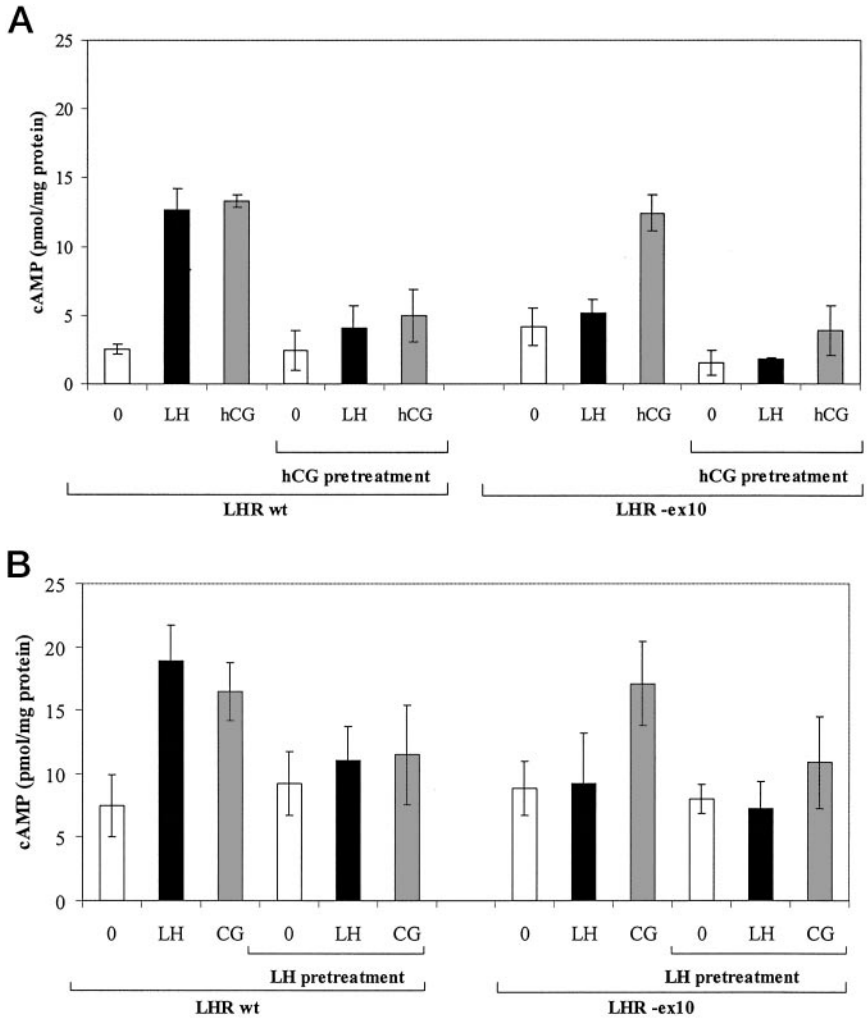

FIG. 7. Receptor uncoupling in LHR wt and LHR –ex10 cell lines. When desensitized with 10^4 IU/liter hCG (a) or LH (b) for 2 h, both cell lines reacted with impaired intracellular cAMP production upon restimulation with 4000 IU/liter LH or hCG, respectively. Compared with control stimulation, intracellular cAMP production by LHR wt cells declined 16% when restimulated with LH and 24% after hCG stimulation (subtracted basal levels). Similarly, in LHR –ex10 cells, cAMP production was not significantly different from the basal level (LH) and decreased by 29% (hCG). For desensitization with LH, the results were similar; intracellular cAMP production by LHR wt cells declined by 16% when restimulated with LH and by 25% when restimulated with hCG (subtracted basal levels). In LHR –ex10 cells, cAMP production was not significantly different from the basal level (LH) and decreased by 35% after hCG stimulation. Results are presented as the mean ± SD of three (a) or two (b) independent experiments determined in triplicate (conversion factor: 1 IU/liter = 5.815 pM).

Discussion

The *in vitro* experiments demonstrate that the human LHR lacking exon 10 has normal binding affinity for LH and hCG, but cAMP production is impaired drastically when stimulated by LH, whereas hCG action is not affected. Furthermore, no increased sequestration of LHR–ex10 inside the cell could be observed. The decrease in LH action on LHR–ex10 cells fully explains the clinical finding of a previously described patient whose LHR lacking exon 10 could not respond to high levels of endogenous LH, but reacted to hCG (14). This requires functional LHRs in sufficient density expressed at the cell surface. In experiments performed with permanently transfected cells we could not confirm the finding by Zhang *et al.* (15), who used transiently transfected 293 cells and reported decreased expression of the LHR lacking exon 10 at the cell surface. The different experimental setting chosen here could justify this discrepancy. However, the observation that LHR lacking exon 10 is normally expressed at the cell surface is consistent with the clinical finding that hCG treatment was effective in the previously described patient (14). Therefore, we cannot share the interpretation by Ascoli *et al.* (9) suggesting that the hypogonadism observed in this patient was ascribed to a reduction in receptor density rather than an impairment of LH action.

The binding experiments performed in this study clearly show that exon 10 of the LHR is not important for binding of hCG and LH, in accordance with previous studies showing that this part of the hinge region is not involved in hCG binding (21). Moreover, our results show that the binding affinities of LH and hCG for the homologous, recombinant LHR permanently expressed in COS-7 cells are significantly different (7-fold). In fact, this difference in binding affinity between the two hormones is evident even when exon 10 is missing. These results are partially at odds with previous data obtained with extractive or recombinant gonadotropins in human corpora lutea, solubilized LHR, or recombinant human LHR (22, 23), but are in agreement with earlier observations suggesting a lower affinity of the human LHR for human LH *vs.* hCG (24). Although our results are very consistent and reproducible, differences in the experimental settings (extracted *vs.* recombinant receptors, transient *vs.* stable transfection, *etc.*) might explain these differences. In any case, our data show that the absence of exon 10 does not significantly change the binding parameters of LH and hCG.

Notwithstanding normal binding characteristics, the LHR lacking exon 10 is less able to induce cAMP production when stimulated by LH and requires about 30-fold higher hormone concentrations to stimulate signal transduction. This impor-

tant finding demonstrates that hormone binding and signal transduction are two distinct properties of the receptor, involving different amino acid regions, as previously shown using LHR mutants (9). Notably, all *in vitro* experiments with LHR mutants performed to date have used hCG, and it would be interesting to investigate whether the findings can be confirmed using LH. This would allow the identification of specific amino acid residues involved in signal transduction common to both hormones.

The binding of LH/hCG to LHR wt leads to activation of the adenylate cyclase-cAMP signaling pathway, resulting in increased cAMP production, activation of adenylate cyclase and protein kinase A, and protein phosphorylation, which finally leads to testosterone production by Leydig cells (25). We speculated that inhibition of adenylate cyclase by LH activation of G_i could occur in the absence of exon 10, but this could be clearly excluded by the experiments using PTX, which could not rescue cAMP production.

Permanent exposure of the LHR to LH/hCG leads to receptor desensitization by uncoupling of the adenylate cyclase or increased activity of the phoshodiesterase that degrades cAMP (25). Thus, another possible explanation for low cAMP formation in response to LH would be a change in such regulatory events of the LHR −ex10 compared with LHR wt. In the experiments presented here, different receptor desensitization is considered a possible reason for the low cAMP production in LHR −ex10 cells stimulated with LH. However, no significant differences in desensitization and recovery of cAMP production in the two cell lines could be observed with hCG or LH desensitization. Thus, different kinetics of receptor desensitization are not involved in the decreased cAMP production by LH in LHR −ex10 cells.

According to the revised ternary model for G protein receptor activation, the LHR exists in a dynamic equilibrium between an inactive (Ro) and an active (R*) state; receptor activation by the hormone results from receptor stabilization in the R* state by removing the constraint of the extracellular domain on the transmembrane domain (26). According to our results, elimination of exon 10 in the hinge region results in reduced ability to stabilize the receptor in the R* state only in the case of LH, not hCG. In the absence of exon 10, LH might act as an inverse agonist and stabilize the receptor in its Ro state. However, the fact that LH does not lose its ability to induce cAMP formation completely, which is possible at much higher LH concentrations, does not support this possibility (27, 28). The C-terminal part of exon 9 comprising the amino acids Thr^{250}-Glu^{268} immediately flanking exon 10 are highly conserved between glycoprotein hormone receptors and species and have been recently demonstrated to be involved in the interaction with hCG, probably by making contact with the α-subunit. Moreover, this region may participate in the interaction of the hCG-exodomain complex with the endodomain, probably via the second extracellular loop of the transmembrane domain (27, 28). In the absence of exon 10, this critical part of the hinge region shifts toward the transmembrane domain, and this displacement is probably sufficient to intercept the interaction of the LH-exodomain complex with exoloop 2. Our data support a model of receptor activation in which it is the hormone itself that activates the serpentine region and suggests that, although not involved in hormone binding, exon 10 is necessary to facilitate contact between LH and residues necessary for receptor stabilization. In other words, the absence of exon 10 could result in a hinge region too rigid or too short to turn the bound LH molecule down to the transmembrane domain. This rigid receptor would not influence the activity of hCG, which, due to its larger dimension, could present the amino acid residues relevant for receptor stabilization and interact with the serpentine region even in the absence of exon 10. It remains to be established whether, for example, the LH-liganded LHR lacking exon 10 might still be able to *trans*-activate neighboring unliganded receptors (29), resulting in cAMP production in the presence of higher hormone concentrations. The concept of LHR *trans*-activation recently proposed by Ji *et al.* (29) implies that one molecule of hormone bound to the extracellular domain of the LHR can activate/stabilize other LHR molecules in its close surroundings, and the researchers show that the cAMP answer of a ligand-defective receptor can be rescued. Cotransfecting the two receptor types investigated here (with or without exon 10) to observe a possible rescue in cAMP production would be a challenging new experiment for the future.

In conclusion, the elimination of exon 10 from the human LHR results in impaired cAMP production by LH in the presence of normal binding, whereas hCG action is not affected. This reduced ability of LH to stimulate such a mutated receptor is not due to inappropriate, intracellular signal transduction events. Rather, the conformational changes induced by normal LH binding to the receptor lacking exon 10 appear to be insufficient or inadequate to stabilize the receptor in its active conformation. This hypothesis awaits confirmation in future studies.

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