The Luteinizing Hormone Receptor Activates Phospholipase C via Preferential Coupling to G_{i2}[†]

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ABSTRACT: Binding of lutropin/choriogonadotropin (LH/CG) to its cognate receptor results in the activation of adenylyl cyclase and phopholipase C. This divergent signaling of the LH receptor is based on the independent activation of distinct G protein subfamilies, i.e., G_s, G_i, and potentially also G_q. To examine the selectivity of LH receptor coupling to phospholipase C β-activating G proteins, we used an in vivo reconstitution system based on the coexpression of the LH receptor and different G proteins in baculovirus-infected insect cells. In this paper, we describe a refined expression strategy for the LH receptor in insect cells. The receptor protein was inserted into the cell membrane at an expression level of 0.8 pmol/mg of membrane protein. Sf9 cells expressing the LH receptor responded to hCG challenge with a concentration-dependent accumulation of intracellular cAMP (EC_{50} = 630 nM) but not of inositol phosphates, whereas stimulation of the histamine H_1 receptor in Sf9 cells led to increased phospholipase C (PLC) activity. Immunoblotting experiments using G protein-specific antisera revealed the absence of quantitative amounts of α_i in Sf9 cells, whereas α_s and $\alpha_{q/11}$ were detected. We therefore attempted to restore the hCG-dependent PLC activation by infection of Sf9 cells with viruses encoding the LH receptor and different G protein α subunits. HCG stimulation of cells coexpressing the LH receptor and exogenous α_{i2} resulted in stimulation of PLC activity. In cells coinfected with an α_{i3}-baculovirus, hCG challenge led to a minor activation of PLC, whereas no hCG-dependent PLC stimulation was observed in cells coexpressing α_{i1}. Most notably, coinfection with baculoviruses encoding α_q or α_{11} did not reproduce the PLC activation by the LH receptor. Thus, the murine LH receptor activates adenylyl cyclase via G_s and PLC via selective coupling to G_{i2}.

The LH[1] receptor plays a crucial role in reproductive physiology. Upon binding to cognate receptors on testicular and ovarian endocrine cells, the glycoprotein hormone LH regulates steroid synthesis and secretion in the gonads (*1*). Together with the FSH and TSH receptors, the LH receptor constitutes the family of glycoprotein hormone receptors (*2*). The LH receptor is a membranous protein exhibiting all the structural hallmarks of a member of the superfamily of G protein-coupled receptors (*3*−*5*). A characteristic feature of the LH receptor is the large extracellular N-terminal domain capable of high-affinity hormone binding (*6*−*8*). The extracellular receptor domain contains N-linked carbohydrate chains of the complex type (*9*, *10*). The question which of the six potential N-linked glycosylation sites are indeed posttranslationally modified is discussed controversially, as well as the impact such glycosylation might have on hormone binding and signal transduction (*11*, *12*).

The LH receptor is able to bind either LH or hCG with high affinity, leading to stimulation of the G_s/adenylyl cyclase system and an increase in the level of cAMP production (*3*, *5*). At high receptor densities and high hormone concentrations, however, the ligand-bound LH receptor additionally initiates phosphoinositide breakdown and Ca^{2+} mobilization (*13*, *14*), thus adding the LH receptor to the list of G protein-coupled receptors with multiple signaling potential (*15*) for which a physiologic role still remains ill-defined. With regard to the mechanism subserving the dual signaling phenomenon, a recent study with bovine luteal cells and L cells permanently expressing the mouse LH receptor revealed that G_s and G_i proteins are directly involved in coupling of the LH receptor to the effector systems adenylyl cyclase and phospholipase C-β, respectively (*16*). G protein-mediated signal transduction can be envisaged as a complex signaling network with convergent and divergent pathways (*15*). Thus, while one heptahelical receptor may be able to interact with different G proteins to elicit multiple intracellular signals, the interaction of a single G protein with a given receptor

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[1] Abbreviations: AC, adenylyl cyclase; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; GPCR, G protein-coupled receptor; G protein, heterotrimeric guanine nucleotide-binding protein; hCG, human chorionic gonadotropin; IBMX, 3-isobutyl-1-methylxanthine; IP, inositol phosphate; IP_3, inositol 1,4,5-trisphosphate; LH, luteinizing hormone; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pfu, plaque forming unit; ni, not infected; PBS, phosphate-buffered saline; pi, post infection; PLC, phospholipase C; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Sf9 cells, cells derived from *Spodoptera frugiperda*; Tris, tris(hydroxymethyl)aminomethane.

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may be subject to a high degree of selectivity (*15*). Alternative splicing gives rise to two $G\alpha_s$ isoforms, whereas three G_i species are encoded by distinct $G\alpha_i$ genes (*17*, *18*). While both G_s isoforms have been found physically associated with the LH receptor (*19*), the identity of the G_i protein interacting with the LH receptor is unknown.

Baculovirus-infected insect cells have been shown to provide a reliable intact cell setting for reconstitution of heptahelical receptors with mammalian G protein subunits (*20*, *21*). Insect cells carry out a variety of posttranslational modifications, albeit not as extensively as mammalian cells, and support targeting of viable receptors and G proteins to the cell membrane. It has been shown that glycoproteins in insect cells carry N-linked carbohydrates of the high-mannose type that are subsequently trimmed to shorter core structures (*22*). So far, no endogenous G protein-coupled receptors which could possibly interfere with exogenously introduced heptahelical receptors have been described in cells derived from *Spodoptera frugiperda* (Sf9).

In the past, biochemical studies on glycoprotein hormone receptor function have been hampered by the limited amounts of receptor that can be obtained from primary tissues. Therefore, baculovirus-based expression systems have been used to acquire substantial quantities of extracellular hormone-binding domain as well as the full-length receptors. Unfortunately, the expression of high levels of functional glycoprotein hormone receptors in baculovirus-infected insect cells has often been tried to no avail because full-length receptors are frequently retained intracellularly (*23*, *24*). All attempts to express the full-length TSH receptor in baculovirus-infected insect cells have failed (*25*, *26*), while expression of a functional FSH receptor has been reported (*27*, *28*). There are contradictory reports about the functional expression of the LH receptor. Infection of Sf9 cells with a baculovirus encoding the complete rat LH receptor led to intracellular trapping of the receptor in one case (*12*), whereas Narayan et al. (*29*) reported the successful cell membrane insertion of the LH receptor in insect cells using the same polyhedrin promoter to drive expression as in the previous study. Incubation of the latter cells with hCG resulted in a moderate rise in intracellular cAMP levels only at relatively high hormone concentrations (*29*). Therefore, it still remains to be shown whether strategies for expressing larger amounts of a fully processed LH receptor with complete signaling potential in insect cells can be devised.

In this paper, we report an optimized expression strategy for a full-length murine LH receptor in baculovirus-infected insect cells. The receptor binds hormone with high affinity and is able to respond to hCG challenge with a concentration-dependent increase in the level of cAMP formation. Restoration of hCG-dependent inositol phosphate formation by coexpression of recombinant $G\alpha_i$ subunits reveals that the murine LH receptor selectively interacts with G_{i2}.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes and DNA-modifying enzymes were from New England Biolabs (Schwalbach, Germany). Lipid concentrate was purchased from Life Technologies (Karlsruhe, Germany), and BaculoGold Baculovirus DNA was from Dianova (Hamburg, Germany). [^{3}H]Pyrilamine (23.4 Ci/mmol), [^{3}H]inositol 1,4,5-trisphosphate (21 Ci/mmol), and Na^{125}I (100 mCi/mL) were from NEN Life Science (Köln, Germany), and [^{3}H]myo-inositol (17.1 Ci/mmol) was from Amersham Buchler (Braunschweig, Germany). HCG was from Calbiochem (Bad Soden, Germany). All other reagents and hormones were from Sigma (Deisenhofen, Germany).

Table 1: Effect of Different MOIs on LH Receptor Expression Levels[a]

MOI (pfu/cell)	expression level (pmol/mg)[b]	MOI (pfu/cell)	expression level (pmol/mg)[b]
3	0.7 ± 0.1	32	0.8 ± 0.1
16	0.6 ± 0.1	48	0.8 ± 0.1

[a] Sf9 cells were infected with a baculovirus encoding the LH receptor cDNA under control of the polyhedrin promoter at MOIs as indicated, and membranes were prepared 72 h pi. Ten micrograms of membrane proteins was incubated with 2 nM [^{125}I]hCG in the presence and absence of 10 μg/mL hCG to determine the levels of unspecific and total binding, respectively. [b] Data are means ± the standard deviation of triplicate determinations and from one representative out of two independent experiments. The expression levels were calculated as total minus unspecific binding.

Methods

Construction of Recombinant Baculoviruses, Insect Cell Culture, and Membrane Preparation. Sf9 cells were maintained as described previously (*30*). The baculovirus encoding the guinea pig histamine H_1 receptor was described previously (*30*). Recombinant baculoviruses encoding the wild-type murine LH receptor were generated as follows. The LH receptor cDNA *Eco*RI fragment from the parent plasmid pBlueSkript Sk$^-$ (*14*) was ligated into the *Eco*RI-linearized transfer vector pVL 1393. A *Not*I−*Xba*I fragment of the LHR pVL 1393 plasmid was then subcloned into the respective restriction sites of pAcMP3. Homologous recombination of viruses and the subsequent plaque assay were performed using standard techniques (*31*). For high-titer virus stocks, Sf9 cells grown in suspension culture were infected at an MOI of 0.2 pfu/cell and grown for 3−4 days. Cells were harvested, and the virus titer of the supernatant was determined by a plaque assay according to standard protocols (*31*). Membrane preparation was carried out according to the methods of Kühn et al. (*32*).

Determination of Infection Efficacy. Sf9 cells were seeded into 35 mm Petri dishes (0.9×10^6 cells/dish) and incubated at 27 °C for 1 h. After removal of the medium, 1 mL of virus stock at the desired MOI was added. Two days thereafter, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the medium according to the manufacturer's instructions (Sigma) to give a final concentration of 0.01 mg/mL. MTT is converted to a purple dye by mitochondrial dehydrogenases of living cells (*33*). The infection efficacy was calculated as the proportion of unstained cells.

Radioligand Binding. Membranes containing the LH receptor were obtained from LHR 11/7 cells (*14*) or from Sf9 cells infected with a virus encoding the murine LH receptor. [^{125}I]hCG was prepared, and saturation binding experiments with increasing concentrations of radioligand were performed to calculate K_d and B_{max} values (*34*). To assess the effect of various MOIs (Table 1) and of different

Table 2: Identification of LH Receptor Binding Sites under Coinfection Conditions[a]

infected baculoviruses	expression level (pmol/mg)[b]	infected baculoviruses	expression level (pmol/mg)[b]
−	0.8 ± 0.1	α_{i2}, β_2, and γ_2	0.7 ± 0.3
α_{i2}	0.7 ± 0.3	α_{11}	0.8 ± 0.1

[a] Sf9 cells were infected with a baculovirus encoding the LH receptor cDNA under control of the polyhedrin promoter and different G protein subunits as indicated in the left column. The MOIs for all baculoviruses were 3 pfu/cell. Seventy-two hours pi, membranes were prepared and 3 μg of membrane proteins was incubated with 2 nM [^{125}I]hCG in the presence and absence of 10 μg/mL hCG to determine the levels of unspecific and total binding, respectively. [b] Data are means ± the standard deviation of triplicate determinations and are from one representative out of two independent experiments. The expression levels were calculated as total minus unspecific binding.

coinfection conditions (Table 2) on LH receptor expression levels, membranes were incubated with 2 nM [^{125}I]hCG in the presence and absence of 10 μg of hCG for unspecific and total binding, respectively. The expression levels determined under these conditions represent total minus unspecific binding. To characterize the H_1 histamine receptor, binding experiments were carried out as described previously (*32*).

Measurement of cAMP Accumulation and Inositol Phosphate Accumulation in Sf9 Cells. Cells were infected and assays were performed as described by Kühn et al. (*32*).

Identification of IP_3 in Sf9 Cells. Cells were infected at an MOI of 3 pfu/cell for each virus, grown for 24−36 h, washed in PBS, resuspended to a density of 5−10 × 10^6 cells/135 μL of PBS supplemented with 10 mM LiCl, and incubated at 27 °C for 10 min. Cells were then challenged with 1 μM hCG, and the reaction was stopped by addition of 300 μL of 50% (w/v) trichloroacetic acid at the indicated times. Levels of IP_3 were measured with a radioreceptor assay using a preparation of bovine liver endoplasmic reticulum as the binding protein (*38*).

SDS−PAGE and Immunoblotting. Membrane proteins were resolved by SDS−PAGE performed on separating gels containing 9% (w/v) acrylamide and 6 M urea (α_i) or 13% (w/v) acrylamide (all other G proteins) and blotted onto nitrocellulose filters. Antisera AS 348, AS 368, AS 266, and AS 11/3 were used for detection of G protein subunits α_s, $\alpha_{q/11}$, α_{i2}, and β, respectively. Antisera were raised against peptide sequences corresponding to specific regions of G protein subunits and have been described previously (refs *35* and *36* for G protein α subunits and ref *37* for β subunits). Immunoreactive bands were visualized as described previously (*35*).

Miscellaneous Methods. Protein concentrations were determined by the bicinchoninc acid protein assay system (Pierce, Rockford, IL). Data were analyzed using the Prism software package (Graph Pad Software).

RESULTS

[^{125}I]hCG Binding to Recombinant LH Receptors. Evidence in the literature suggests that the yield and viability of expression of glycoprotein hormone receptors in insect cells depend on the type of baculoviral promotor which is used to drive the expression of the cDNA (*39*, *40*). We therefore approached the expression of a functional LH receptor by generating two different recombinant baculoviruses using either the late basic protein promoter (pAcMP 3) or the very late polyhedrin promoter (pVL1393) to drive the expression of the LH receptor. Using a [^{125}I]hCG binding assay (*34*), LH receptor expression under control of the basic protein promoter could be detected 12 h pi and peaked 36 h pi at a B_{max} of 199 ± 11 fmol/mg (Figure 1A), whereas expression controlled by the stronger polyhedrin promoter could be detected 24 h pi and was maximal 96 h pi at a B_{max} of 764 ± 21 fmol/mg (Figure 1B). When LH receptor expression was driven by the late basic promoter (pAcMP 3), saturation binding experiments allowed us to calculate a mean K_d value of 275 ± 60 pM [mean ± the standard error of the mean (SEM), $n = 5$], while the use of the very late polyhedrin promoter entailed a slightly reduced binding affinity (K_d = 345 ± 63 pM, $n = 7$). Uninfected cells exhibited no significant specific [^{125}I]hCG binding. As the polyhedrin promoter-driven expression generated more hCG-binding sites and because binding affinities were comparable, we decided to continue this study making use of the polyhedrin promoter.

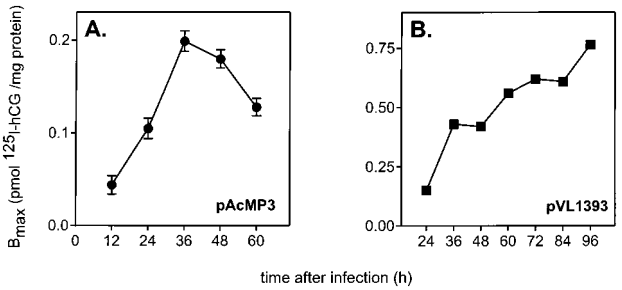

FIGURE 1: Time course of expression of the LH receptor under control of the basic protein promoter (A, pAcMP3) and under control of the polyhedrin promoter (B, pVL1393). Sf9 cells were infected with baculoviruses encoding the LH receptor under the control of the basic protein (A) or under control of the polyhedrin (B) promoter at an MOI of 3 pfu/cell, and membranes were prepared at the indicated times. Ten micrograms of membrane protein was incubated with increasing concentrations of [^{125}I]hCG in the presence or absence of 10 μg/mL hCG to determine the levels of unspecific and total binding, respectively. The B_{max} values were calculated by nonlinear regression analysis. Data are means ± the standard deviation from one of three independent experiments, each performed in triplicate.

We also determined the effect of the concentration of the recombinant baculovirus (MOI) on the expression of [^{125}I]-hCG binding sites. Sf9 cells were infected at different MOIs with the baculovirus encoding the LH receptor cDNA under control of the polyhedrin promoter. Membranes were prepared 72 h pi and subjected to [^{125}I]hCG binding (Table 1). At an MOI of 3 pfu/cell, membranes from infected Sf9 cells already contained the LH receptor at fairly high levels (see Table 1). Application of baculoviruses at higher MOIs did not lead to a further increase in the levels of [^{125}I]hCG binding.

We next set out to determine the binding characteristics of the LH receptor in Sf9 cells. Therefore, membranes were prepared from Sf9 cells expressing the LH receptor and from LHR 11/7 cells and subjected to [^{125}I]hCG binding (Figure 2). Scatchard analyses indicated the presence of 478 and 741 fmol of [^{125}I]hCG binding sites per milligram of membrane protein in LHR cell membranes and Sf9 cell membranes, respectively. The apparent dissociation constant (K_d) was

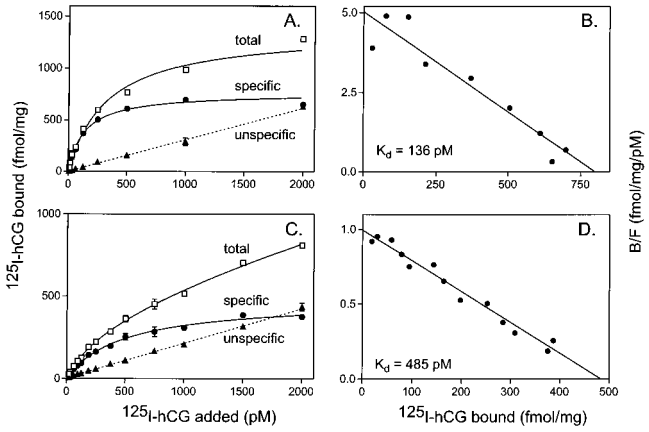

FIGURE 2: Saturation binding experiments with Sf9 and L cell membranes expressing the LH receptor. (A) Membranes from Sf9 cells expressing the LH receptor under the control of the polyhedrin promoter were prepared 72 h pi. Ten micrograms of the membrane protein was incubated with increasing concentrations of [^{125}I]hCG. (C) Membranes (4 μg) from L cells stably expressing the LH receptor were incubated with increasing concentrations of [^{125}I]-hCG. The levels of unspecific and total binding were determined in the presence and absence of 10 μg/mL hCG, respectively. Panels B and D are Scatchard transformations of data depicted in panels A and C, respectively. Data are means ± the standard deviation of triplicate determinations from one of three independent experiments.

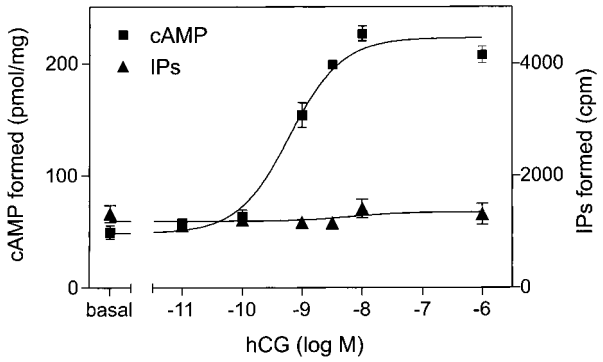

FIGURE 3: Functional coupling of the LH receptor to adenylyl cyclase in baculovirus-infected Sf9 cells. Cells were infected with a virus encoding the LH receptor under the control of the polyhedrin promoter at an MOI of 3 pfu/cell and were labeled with 2 μCi/mL of [^{3}H]myo-inositol for determination of the level of inositol phosphates. Cells were aliquoted to a density of 0.5×10^6 per tube and stimulated with hCG in the presence of 1 mM IBMX or 10 mM LiCl for 10 min for elucidation of cAMP or inositol phosphate accumulation, respectively. Levels of cAMP were determined by radioimmunoassay, and inositol phosphates were separated by anion exchange chromatography. Data are from one of three representative experiments and are means ± the standard deviation of triplicate determinations. IP represents inositol phosphate.

higher in membranes of LHR 11/7 than in Sf9 cells (see Figure 2). To calculate the number of [^{125}I]hCG binding sites per cell, we determined the infection efficacy, i.e., the percentage of infected cells at a given MOI. To this end, we stained infected and noninfected cells with MTT. An infection efficacy of 75% when using an MOI of 3 pfu/cell allowed us to determine that 20000−30000 [^{125}I]hCG binding sites were present in each Sf9 cell.

hCG-Dependent cAMP Formation but a Lack of Stimulation of Phospholipase C in Sf9 Cells. The signaling potential of the LH receptor in Sf9 cells was tested in cells that were infected with a baculovirus (MOI = 3 pfu/cell) encoding the LH receptor under control of the polyhedrin promoter. Stimulation of baculovirus-infected Sf9 cells with hCG led to concentration-dependent cAMP accumulation in the presence of 1 mM IBMX (Figure 3). The concentration for half-maximal activation (EC_{50}) of adenylyl cyclase was 630 ± 11 pM hCG (mean ± SEM, n = 3). Whereas hCG stimulation led to a 6-fold increase in the extent of cAMP accumulation, we did not observe an hCG-induced stimulation of phospholipase C activity (see Figure 3).

To test the hypothesis that the inability to demonstrate coupling of the LH receptor to phospholipase C might be due to functional constraints inherent to the recombinant model system which was chosen, the H_1 histamine receptor, which increases PLC activity by G proteins of the $G_{q/11}$ subfamily, was also expressed and studied in Sf9 cells. Binding of the antagonist [^{3}H]pyrilamine to membranes prepared from Sf9 cells which expressed the guinea pig H_1 histamine receptor was specific and saturable (Figure 4A). Scatchard analysis of these data revealed the presence of 3.0 ± 0.3 pmol (mean ± SEM, n = 3) of [^{3}H]pyrilamine binding sites per milligram of membrane protein and a dissociation constant for [^{3}H]pyrilamine of 1.5 ± 0.5 nM. These results allowed us to calculate a density of 83 000 [^{3}H]pyrilamine binding sites per Sf9 cell. Histamine challenge of those cells led to an increase in the level of inositol phosphate formation in a concentration-dependent manner, which was inhibited by 10 μM diphenhydramine (Figure 4B).

Analysis of G Protein α and β Subunit Expression in Sf9 Cells. It has previously been reported that the G_i subtype of G proteins is only expressed in trace amounts (*41*) or might even be absent (*21*, *42*−*46*) in Sf9 cells. Whereas several groups reported the presence of pertussis toxin-sensitive G proteins of the G_o subtype (*21*, *41*, *42*), others could not detect G_o proteins (*43*, *44*). To test the hypothesis that the absence of G proteins of the G_i subfamily might uncouple the LH receptor from activation of PLC, we first analyzed the G protein contents of our Sf9 cell strain via immunoblotting experiments. In membranes of noninfected Sf9 cells, an antiserum raised against a carboxy-terminal peptide sequence of α_s (AS 348) stained four prominent and several faint bands migrating above the 43 kDa marker protein. Blocking experiments using the peptide that was selected to generate the antibody revealed those bands which were specifically recognized: a doublet located slightly above the 43 kDa marker and an additional band with an apparent molecular mass of about 46 kDa (Figure 5A, left-most and right-most lanes). In membranes of Sf9 cells expressing the cDNA corresponding to the long splice variant of α_s, the immunoreactivity of a prominent band of about 46 kDa was increased, matching the upper band in membranes of noninfected cells (see Figure 5A, center lane).

An antiserum specific for α_q and α_{11} (AS 368) showed the expression of a 42 kDa protein in membranes prepared from noninfected Sf9 cells (Figure 5B, left-most lane). This band could be effectively competed for by addition of the peptide which was used to generate the antiserum. In membranes prepared from Sf9 cells expressing α_{11} (see Figure 5B, center lane), we detected a prominent specific band at approximately 42 kDa. In addition, two fainter bands were present in the 50 and 90 kDa range, which might be explained by aggregate formation as described for recombinant α_q (*47*) and α_{11} (*48*).

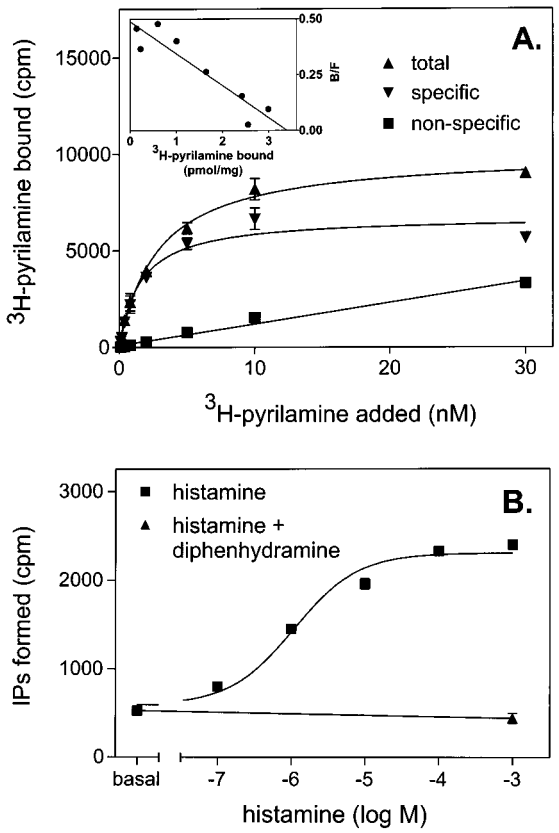

FIGURE 4: Expression of the histamine H_1 receptor in Sf9 cells. (A) Saturation binding of [^{3}H]pyrilamine to membranes (80 μg per tube) of Sf9 cells expressing the H_1 receptor is depicted. Cells were infected with recombinant baculovirus at an MOI of 3 pfu/cell and grown for 48 h. Membrane preparation and radioligand binding were performed as described in Methods. Data are means ± the standard error of the mean of triplicate determinations. Results from one of three representative experiments are shown. The inset shows the Scatchard transformation of the data. (B) Agonist-induced inositol phosphate formation via the H_1 histamine receptor expressed in Sf9 cells. Cells were infected with a virus encoding the H_1 histamine receptor under the control of the polyhedrin promoter at an MOI of 3 pfu/cell and were labeled with 2 μCi/mL of [^{3}H]-myo-inositol. Cells were aliquoted to a density of 0.5×10^6 per tube and stimulated for 10 min with histamine in the presence of 10 mM LiCl (■) and additionally with 10 μM diphenhydramine (▼). Inositol phosphates were separated by anion exchange chromatography. Data are from one of three representative experiments and are means ± the standard deviation of triplicate determinations.

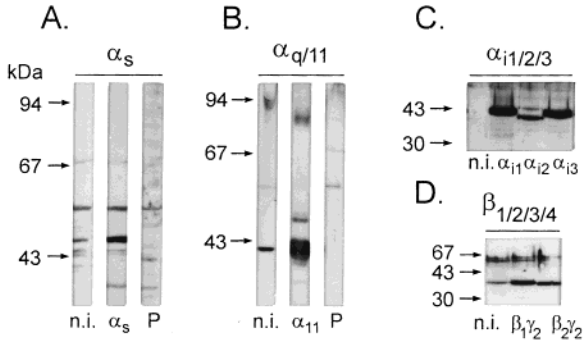

FIGURE 5: Immunoblot analysis of G proteins in baculovirus-infected Sf9 cells. (A) Membrane proteins of noninfected Sf9 cells (100 μg per lane) and membranes of Sf9 cells expressing the LH receptor and recombinant α_s (10 μg per lane) were resolved by SDS−PAGE [13% (w/v) acrylamide]. (B) Membrane proteins of noninfected Sf9 cells (100 μg per lane) and membranes of Sf9 cells expressing the LH receptor and recombinant α_{11} (5 μg per lane) were resolved by SDS−PAGE [13% (w/v) acrylamide]. (C) Membrane proteins of noninfected Sf9 cells (100 μg per lane) and membranes of Sf9 cells expressing the LH receptor and recombinant α_i (50 μg per lane) were resolved on SDS gels containing 9% (w/v) acrylamide and 6 M urea. (D) Membrane proteins (20 μg per lane) of noninfected Sf9 cells and membranes of Sf9 cells expressing the LH receptor and recombinant α_{i2} and β_1 and γ_2 or β_2 and γ_2 were resolved by SDS−PAGE [13% (w/v) acrylamide]. Filters were cut into strips that were incubated with the antisera as indicated at the top [α_s (AS 348, 1/150), $\alpha_{q/11}$ (AS 368, 1/150), $\alpha_{i1/2/3}$ (AS 266, 1/150), and $\beta_{1/2/3/4}$ (AS 11, 1/300)] or with the antiserum and the peptide (10 μg/mL) that was used to generate the serum. Molecular masses (kilodaltons) of the standard marker proteins are indicated on the left. Abbreviations on the bottom of each lane indicate infection conditions or peptide block: ni, noninfected; α_s, α_{11}, α_{i1}, α_{i2}, and α_{i3}, infected with viruses encoding the LH receptor and α_s, α_{11}, α_{i1}, α_{i2}, and α_{i3}, respectively; $\beta_1\gamma_2$ and $\beta_2\gamma_2$, cells infected with viruses encoding the LH receptor, α_{i2}, and $\beta_1\gamma_2$ or $\beta_2\gamma_2$; P, peptide block.

An antibody recognizing all three α_i subunits (AS 266) allowed us to assess the presence of α_i subunits in Sf9 cells. Even though maximal amounts of membrane protein (100 μg) were loaded, we were not able to detect immunoreactive proteins in membranes prepared from noninfected Sf9 cells (Figure 5C, left-most lane), whereas in 50 μg of membranes from Sf9 cells that were infected with viruses encoding either α_{i1}, α_{i2}, or α_{i3}, immunoreactive bands with the appropriate molecular mass could be readily detected (see Figure 5C, three right-most lanes). As expected, α_{i2} migrated slightly faster than α_{i1} and α_{i3} (*35*). Thus, in addition to α_s-like G proteins, Sf9 cells also contain α_q-like G proteins, whereas no endogenous α subunits belonging to the G_i subfamily could be detected under the experimental conditions that were chosen.

Furthermore, immunoblotting with an antibody directed against a peptide sequence of G protein β subunits 1−4 (AS 11) revealed a single band in the 36 kDa range in uninfected Sf9 cells (Figure 5D, left-most lane). Membranes prepared from Sf9 cells expressing either β_1 and γ_2 or β_2 and γ_2 exhibited substantially increased immunoreactivity with the anti-$\beta_{1/2/3/4}$ antiserum when compared to noninfected cells (see Figure 5D, two right-most lanes). Taken together, these data demonstrate that Sf9 cells express the signal transduction machinery necessary for signal transmission from heptahelical receptors to PLC via G proteins of the $G_{q/11}$ subfamily.

In Vivo Reconstitution of the LH Receptor and Different G Protein α *and* $\beta\gamma$ *Subunits.* To prove our hypothesis that the low expression level of G proteins uncouples the activated LH receptor from stimulation of PLC, we attempted the functional reconstitution of this signal transduction cascade by infection with baculoviruses encoding different G protein α subunits. Infection of Sf9 cells with baculoviruses encoding various subunits of mammalian heterotrimeric G proteins leads to efficient expression of α, β, and γ subunits (*47*, *49*, *50*). Furthermore, simultaneous expression of receptor and G protein cDNA is a feasible approach to studying receptor−G protein interaction (*20*, *21*, *47*, *51*). Sf9 cells were thus simultaneously infected with baculoviruses encoding the cDNA of the LH receptor and of different G protein α subunits, and their expression was confirmed by immunoblotting (see Figure 5A−C, center lanes).

Previous reports indicate that expression levels of receptor and G protein influence each other reciprocally (*51*). We therefore assessed the level of [^{125}I]hCG binding sites in membranes of Sf9 cells expressing the LH receptor and

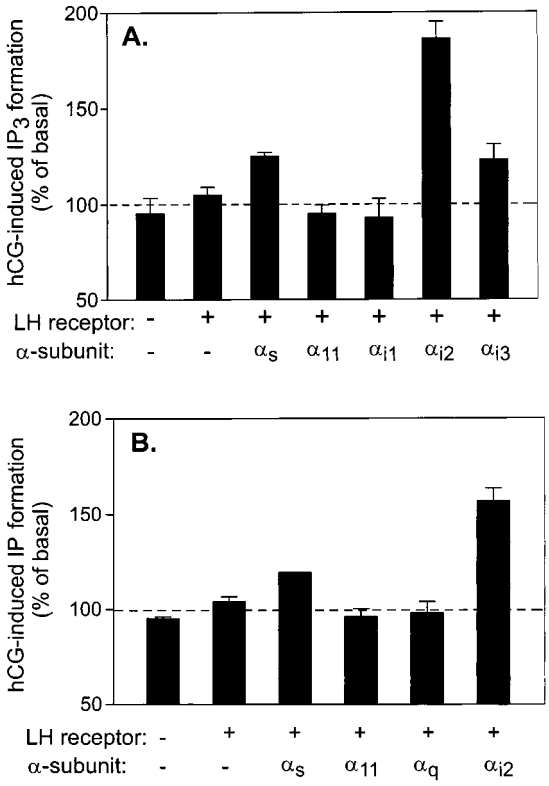

FIGURE 6: HCG-induced inositol 1,4,5-trisphosphate (A) and inositol phosphate (B) formation in baculovirus-infected insect cells. (A) Sf9 cells were infected with baculoviruses as indicated and grown for 24−36 h pi, washed, and stimulated with 1 μM hCG in aliquots of 5−10 $\times$ 10^6 cells per tube. The reaction was stopped by addition of trichloroacetic acid 15 s after addition of 1 μM hCG. Levels of IP_3 were determined by a radioreceptor assay. The dashed line represents the level of IP_3 formation in the absence of hCG. (B) Sf9 cells were infected with the viruses as indicated, labeled with 2 μCi/mL of [^{3}H]myo-inositol for 36 h, and then incubated with 10 mM LiCl for 10 min. Aliquots of 0.5 $\times$ 10^6 cells were stimulated with 1 μM hCG for 10 min. Levels of inositol phosphates were determined by anion exchange chromatography. The dashed line represents the level of IP formation in the absence of hCG. Data are means $\pm$ the standard error of the mean of three independent experiments, each performed in triplicate. IP_3 represents inositol 1,4,5-trisphosphate and IP inositol phosphates.

different complements of G protein subunits (Table 2). Under these conditions, infection of Sf9 cells with more than one virus did not suppress the level of [^{125}I]hCG binding sites below 0.7 pmol/mg.

To analyze a potential hCG-dependent stimulation of PLC via G_i proteins, Sf9 cells were infected with a baculovirus encoding the LH receptor and different baculoviruses encoding various G protein α subunits, and hCG-stimulated PLC activation was assessed 24−36 h pi. Sf9 cells solely expressing the LH receptor exhibited no significant degree of hCG-stimulated IP_3 and IP formation when compared to noninfected cells (Figure 6A,B), whereas coinfection with a baculovirus encoding α_{i2} increased the level of hCG-stimulated inositol phosphate production to about 180% of the baseline value. Coinfection with a virus encoding α_{i3} or α_s also resulted in a small increase in the level of hCG-dependent IP_3 formation, whereas coinfection with viruses encoding either α_{11} or α_{i1} had no effect on the activation of PLC by the LH receptor (see Figure 6A). The coupling of the LHR to G_{i2} was confirmed in yet another set of experiments (see Figure 6B). Thus, infected Sf9 cells were

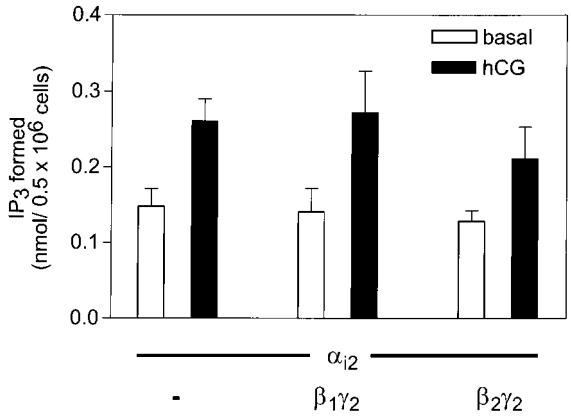

FIGURE 7: HCG-induced inositol 1,4,5-trisphosphate formation in infected Sf9 cells. Sf9 cells were infected with baculoviruses encoding the LH receptor and different G proteins as indicated. Cells were grown for 24−36 h pi and washed, and 5−10 $\times$ 10^6 cells per tube were stimulated with 1 μM hCG. The reaction was stopped 15 s later by addition of trichloroacetic acid. Levels of IP_3 were determined as described in Methods. Data are means $\pm$ the standard error of the mean of three independent experiments, each performed in triplicate.

labeled with [^{3}H]myo-inositol, and hCG-stimulated IP formation was assessed. Cells coexpressing the LHR and α_{i2} responded with increased levels of IP formation (see Figure 6B). Furthermore, coinfection with a virus encoding α_q or α_{11} did not reproduce the hCG-dependent IP formation, whereas coinfection with α_s resulted in a slightly increased level of IP formation, comparable to that determined with the radioreceptor assay (cf. Figure 6A,B).

We furthermore tested the hypothesis that additional infection with viruses encoding G protein $\beta\gamma$ subunits might enhance the hCG-stimulated IP_3 formation. Therefore, Sf9 cells were infected with baculoviruses encoding the LH receptor, α_{i2}, β_1 and γ_2, or β_2 and γ_2, and expression of β subunits was confirmed by immunoblotting (see Figure 5D). Cells infected with viruses encoding the LH receptor and the α_{i2} subunit served as a control and exhibited a 50% increase in the level of hCG-stimulated IP_3 formation. Coexpression of either $\beta_1\gamma_2$ or $\beta_2\gamma_2$ did not lead to a further increase in the level of hCG-dependent IP_3 formation (Figure 7).

DISCUSSION

The existence of a cAMP-independent signaling pathway for the LH receptor has been well established (*1*). Several groups have demonstrated that the stimulated LH receptor can also activate PLC in rat granulosa cells (*52*), in bovine corpus luteum cells (*13*), and in L cells stably transfected with the murine LH receptor (*14*). Generally, receptor-induced stimulation of PLC has been shown to occur via PTX-insensitive $G\alpha_{q/11}$ or via $G\beta\gamma$ from activated PTX-sensitive G_i (reviewed in refs *53* and *54*). In a recent study, we identified the activation of G_s and G_i as the first steps of the LH receptor-stimulated cellular signal transduction cascade in LHR cells as well as in bovine corpus luteum (*16*). The functional consequence of hCG-dependent stimulation of G_i in L cells is activation of PLC and possibly inhibition of adenylyl cyclase. Another study in which porcine follicular membranes were used showed that the long and short variants of G_s and also G_i were activated upon

stimulation with hCG (*19*). Furthermore, in a consecutive study, hCG stimulation of porcine follicular membranes led to additional activation of G_{13} and $G_{q/11}$ (*55*). Most notably, receptor-stimulated binding of [α-^{32}P]GTP azidoanilide to $G\alpha_{q/11}$ proteins in ovarian follicular membranes proceeded with an exceptionally slow time course (*55*) when compared to those of other receptors interacting with $G_{q/11}$ proteins (*56*). Furthermore, hCG-dependent PLC activation cannot be detected in these membranes, which does not support a physiological function of hCG-dependent G_q activation in porcine follicular membranes (*55*). The extended coupling pattern of the LH receptor in porcine follicular membranes compared to that of the bovine corpus luteum might be attributable to species differences or to the use of different cellular systems. Interestingly, the LH receptor in MA-10 Leydig tumor cells activates adenylyl cyclase but not phospholipase C (*57*).

Since the G_i subfamily of G proteins consists of at least six different gene products, among them G_{i1}, G_{i2}, and G_{i3} (*17*), it was difficult to assign the stimulation of a particular $G_{i/o}$ protein to the activated LH receptor. In our previous study (*16*), we have shown that the LH receptor activates members of the G_i subfamily, which were not specified further. By using PTX-dependent ADP ribosylation and photolabeling with [α-^{32}P]GTP azidoanilide, hCG-dependent labeling of a single band, which represented G_i, was detected by Hunzicker-Dunn and colleagues (*19*). However, this band was not further characterized (*19*, *55*). At this point, it was not clear whether the LH receptor was capable of activating all of the members of the G_i subfamily. The purpose of the study presented here was to identify the particular members of the G_i subfamily which couple the agonist-bound LH receptor to PLC using the approach of in vivo reconstitution of receptor and G proteins in baculovirus-infected Sf9 cells.

The baculovirus/insect cell system has become a powerful tool for functional and high-level expression of various signal transduction components, including GPCRs. Several groups reported the expression of GPCRs belonging to the subfamilies of aminergic and peptidergic receptors (*21*, *58*−*60*). To achieve a high level of LH receptor expression, it was necessary to define optimal conditions for the production of biologically active receptors. We approached the high-level expression of a functional LH receptor by using two different baculovirus transfer vectors for the generation of recombinant viruses. Expression of the LH receptor cDNA under control of the very late polyhedrin promoter yielded a higher level of expression of the receptor than when using the basic protein promoter instead of the polyhedrin promoter (764 and 199 fmol of [^{125}I]hCG binding sites per milligram, respectively). Interestingly, expression of the TSH receptor ectodomain in Sf9 cells yielded a secreted protein capable of neutralizing TSH receptor autoantibodies and inhibition of TSH binding only when expressed under the control of the late basic protein promoter (*40*).

The level (B_{max}) of LH receptor expression in membranes obtained from Sf9 cells infected with the virus encoding the LH receptor cDNA under control of the polyhedrin promoter (741 fmol/mg) is comparable to that determined for recombinant mammalian systems and bovine corpora lutea (*61*). The affinities (K_d) of [^{125}I]hCG for the LH receptor expressed in Sf9 cells and in LHR cells were comparable, thus indicating the expression of a functional receptor in both cellular systems. However, there may be a difference in posttranslational modification, since insect cells glycosylate the LH receptor with a high number of mannose residues (*12*), whereas in mammalian cells, proteins are glycosylated with complex carbohydrates (*40*).

The density of the LH receptor which was achieved is rather low compared to those of other receptors expressed in insect cells despite using the strong polyhedrin promoter to drive the expression (*51*, *60*). Most probably, the size and the complex structure of the LH receptor are responsible for the considerably lower expression level. It is also interesting to note that coinfection with more than one virus did not attenuate the number of [^{125}I]hCG binding sites as was reported for the D_{2S} receptor (*51*). This might be due to the expression level of the LH receptor being 50-fold lower than that of the D_{2S} receptor (*51*). Interestingly, the level of endogenous α_s and $\alpha_{q/11}$ in Sf9 cells infected with the histamine H_2 receptor was not significantly diminished at the time points chosen for second-messenger assays (*32*).

The activation of two independent second-messenger cascades in insect cells, i.e., increase in cAMP and IP levels, by the histamine H_2 receptor has been demonstrated previously (*32*). Since the LH receptor is a classical G_s-coupled receptor (*14*), we first studied the hCG-stimulated cAMP formation in baculovirus-infected Sf9 cells and obtained a concentration-dependent increase in the level of intracellular cAMP with a half-maximal stimulation at a concentration (EC_{50}) of 623 pM hCG, similar to that obtained from rabbit corpus luteum (*34*). These results demonstrate the expression of the murine LH receptor as a functionally active protein. Surprisingly, we were not able to detect hCG-stimulated phosphatidylinositol hydrolysis in Sf9 cells expressing the LH receptor alone, whereas Sf9 cells expressing the H_1 histamine receptor responded to histamine stimulation with inositol phosphate formation and a subsequent increase in the intracellular Ca^{2+} concentration (*32*). We thus concluded that the LH receptor and the histamine H_1 receptor signaling to PLC by G proteins might employ two distinct mechanisms, for instance, different G protein subfamilies.

Our immunoblotting experiments and previous reports (*44*) showed that endogenous α subunits belonging to the G_i subfamily were not expressed in appreciable amounts in insect cell membranes, whereas α subunits of the G_s and $G_{q/11}$ subfamilies were abundant (*32*). We thus hypothesized that the lack of detectable amounts of endogenous G_i proteins might be responsible for the absence of hCG-stimulated PLC activity in Sf9 cells, whereas the histamine-dependent increase in the level of phosphatidylinositol hydrolysis was unaffected due to the presence of $G_{q/11}$. However, odorant receptors which activate PTX-sensitive $G_{i/o}$ proteins in mammalian cells (*62*) have been shown to stimulate PLC in Sf9 cells (*63*). Thus, there is reason to assume that odorant receptors recombinantly expressed in Sf9 cells stimulate PLC via G_o proteins which were detected in Sf9 cells (*21*, *41*, *42*). The dual coupling potential of GPCRs is dependent on receptor density (*64*) which might explain the functional presence of only the G_s/adenylyl cyclase system at low receptor densities. However, the number of [^{125}I]hCG binding sites in Sf9 cells was 20000−30000 per cell which is well beyond the threshold of 500−4000 receptors for activation of PLC in LHR 11/6 cells (*64*).

The baculovirus/insect cell system offers the possibility of coexpressing proteins by simultaneous infection with different viruses (*21*), which enabled us to study the influence of different G protein α subunits on hCG-dependent PLC activation. Grünewald et al. (*51*) applied the same approach to show the preference for coupling of the dopamine D_{2S} receptor to G_{i1} over coupling to G_{i2}. Toward this end, we coinfected Sf9 cells with viruses encoding the LH receptor and different α subunits. Only cells expressing the LH receptor and α_{i2} responded to hCG stimulation with an increase in PLC activity, whereas coinfection of α_{i3} and α_s had only minor effects. Activation of PLC via G_s by an ill-understood mechanism has previously been reported for *Xenopus* oocytes (*65*). In view of the possibility that G protein $\beta\gamma$ subunits can transduce the signal for Ca^{2+} release in *Xenopus* oocytes (*66*), one may speculate that $\beta\gamma$ subunits released from G_s can also contribute to phospholipase C activation, particularly when G_s and a receptor efficiently coupling to this G protein are overexpressed.

The hCG-induced PLC activation is smaller than the one elicited by the $G_{q/11}$-coupled H_1 histamine receptor and thus reliably mimics the situation in mammalian cells (*14*, *16*). In this respect, it has to be kept in mind that in the Sf9 cell system agonist binding to $G_{q/11}$-coupled receptors only leads to an approximately 3-fold increase in the level of inositol phosphate production even at optimal time points pi (see Figure 4B; *58*). Interestingly, coexpression of the LH receptor and α_q (see Figure 6B), α_{11}, or α_{i1} (see Figure 6A) did not enhance hCG-stimulated phosphatidyl inositol hydrolysis, although receptor expression levels were constant under the coinfection conditions that were employed. The observation that the LH receptor does not couple well to a heterotrimer containing α_{i1} is in keeping with the observation that the LH receptor, α_{i2}, and α_{i3} are components of a functional signaling axis in vivo, whereas the expression of α_{i1} is limited to neuronal tissue (*67*). However, preferential coupling to α_{i2} versus α_{i3} emphasizes the remarkable specificity of LH receptor−G protein interaction, which may also be important in the physiologic environment of the LH receptor (*16*).

There is inconsistency in the literature with respect to whether, for in vivo signal transduction studies in Sf9 cells, it is mandatory to express all of the components of the receptor/G protein signal transduction cascade. Hepler et al. (*17*) observed an efficient heterotrimerization of heterologously expressed recombinant α and endogenous $\beta\gamma$ subunits in insect cells by copurification. Expression of the 5-HT_{1A} receptor with the G protein subunits α_i, β_1, and γ_2 reconstituted more high-affinity agonist binding sites than expression of the 5-HT_{1A} receptor and α_i alone (*21*), whereas serotonin-dependent [^{35}S]GTPγS binding to activated G_z was not different when the 5-HT_{1A} receptor was expressed with α_z alone or together with α_z, β_1, and γ_2 (*20*). Furthermore, the m_1-muscarinic acetylcholine receptor activated PLC in insect cells expressing recombinant α_q (*68*). Because additional infection with viruses for β_1 or β_2 and γ_2 failed to further increase the level of hCG-stimulated PLC activation, one may speculate that the recombinantly expressed α subunits either effectively assembled with endogenous Sf9 cell $\beta\gamma$ complexes or that the expression of two additional subunits caused a corresponding suppression of α_{i2} levels. In additional studies with the β_2-adrenergic receptor expressed in Sf9 cells, we could rule out the latter possibility (B. Kühn and T. Gudermann, unpublished results).

Activating mutations of α_{i2} have been identified in a subset of ovarian sex cord stromal tumors and adrenal cortical tumors (*69*). By demonstrating preferential G_{i2} coupling of the activated LH receptor, our studies may be helpful in deciphering the signaling pathways leading to LH/CG-dependent growth and differentiation in the gonads.

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