Involvement of Gα and Gβ Proteins in Dual Coupling of the Luteinizing Hormone Receptor to Adenylyl Cyclase and Phospholipase C*

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Binding of lutropin/choriogonadotropin to its cognate receptor results in the activation of adenylyl cyclase and phospholipase C. The mechanism underlying the generation of this bifurcating signal is presently not known. To analyze the coupling mechanism of the LH receptor, activated G proteins were labeled with [α-32P]GTP azidoanilide and identified by selective immunoprecipitation. In membranes of bovine corpora lutea and of L cells stably expressing the murine LH receptor (LHR cells), human chorionic gonadotropin (hCG) led to incorporation of the label into α2 and α5. Stimulation of LHR cells or of L cells expressing the M5 muscarinic receptor (L5MS cells) with the respective agonist resulted in activation of phospholipase C in both cell lines. However, α5 and α5 were only labeled upon stimulation of the M5 muscarinic receptor. Agonist-induced Ca2+ mobilization and inositol phosphate accumulation were partially sensitive to pertussis toxin, and the expression of the βγ-stimulable phospholipase C isoforms β2 and β3 could be demonstrated in LHR cells. Overexpression of phospholipase C-β2 led to increased hCG-stimulated inositol phosphate accumulation, and expression of a β-ARK1 C-terminal polypeptide effectively suppressed hCG-mediated phosphatidylinositol hydrolysis. Thus, the LH receptor couples to both Gα and Gβ and Gβ-subunits released from either G protein contribute to the stimulation of phospholipase C-β isoforms.

The glycoprotein hormone luteinizing hormone (LH) binds to a specific cell surface receptor on testicular Leydig cells and on ovarian theca, interstitial, luteal, and mature granulosa cells to regulate steroid synthesis and secretion in the gonads (1). Cyclic AMP has long been shown to be a key second messenger, being generated upon LH binding to its receptor; however, there is evidence that multiple signal transduction pathways are involved in mediating the cellular actions of LH and hCG (1). For both luteal and granulosa cells it has been reported that stimulation with LH or hCG results in PLC activation, and protein kinase C signaling following LH receptor activation in a number of gonadal steroidogenic cells has been reported. The LH surge is the obligatory physiological signal for ovulation and appears to activate not only the cAMP/protein kinase A pathway but also the protein kinase C pathway (2). In L cells, Xenopus oocytes, and HEK 293 cells, the cloned LH receptor has been shown to activate PLC in addition to its ability to stimulate adenylyl cyclase (3–5). This does not appear to be the case for all cell lines expressing the LH receptor, as an hCG-induced increase in inositol phosphates and/or diacylglycerol could not be observed in Leydig tumor cells (6, 7), and LH stimulation did not lead to mobilization of cytosolic Ca2+ in single rat Leydig cells (8). The reasons for these discrepant results are unknown.

The fact that a single ligand elicits changes in more than one second messenger system is not unprecedented. Several cloned receptors have been described that mediate inhibition of adenylyl cyclase and stimulate PLC (summarized in Ref. 9). As shown for the M2 muscarinic and the 5-HT1A receptors, both signaling pathways are sensitive to PTX and thus are both mediated by a G protein belonging to the Gβ family. Activated α2-subunits are thought to mediate inhibition of adenylyl cyclase, whereas PLC-β isoforms (10) are assumed to be activated by βγ-subunits released from activated Gβ. A second type of dual signaling has been described for receptors that stimulate both adenyllyl cyclase and PLC. Among these are two more glycoprotein hormone receptors, the thyroid-stimulating (11) and the follicle-stimulating hormone receptors (12), several members of the glucagon/secretin receptor family, the D1 dopamine and turkey β-adrenergic receptors (see Ref. 9 and references therein). In a recent study, overexpression of the human β1- or β2-adrenergic or of the V2 vasopressin receptors also led to stimulation of PLC at high agonist concentrations (13). Comparable with the situation with Gβ-coupled receptors, PLC activation by Gβ-coupled receptors requires higher agonist concentrations as opposed to stimulation of adenylyl cyclase.

Activation of adenylyl cyclase and PLC by Gα-coupled receptors has been interpreted either in terms of coupling to two different G proteins (Gα and Gβγ) or in terms of generation of GTP-bound α5 and βγ dimers derived from Gα. For native human thyroid membranes, it has been reported that the thyroid-stimulating hormone receptor couples to G proteins of both the Gα and Gγ families (15). It is not clear, however, whether this coupling principle can be extended to other Gα-coupled receptors. In the present study, we critically addressed this issue by characterizing the G protein-coupling pattern of the closely related LH receptor in L cells stably transfected with the cDNA coding for the cloned murine receptor as well as in bovine corpus luteum membranes, which endogenously express the LH receptor.
**EXPERIMENTAL**

**Materials**

Radiochemicals—\(^{125}\text{I}
\)TTP-GTP (3000 Ci/mmol), \(^{32}\text{P}
\)3-deoxy-ATP (2000 Ci/mmol). \(^{3}\text{H}
\)prolidine \(E_{\text{r}}\), and carrier-free Na\(^{22}\) were purchased from DuPont NEN (Bad Homburg, Germany). myo-[\(^{3}\text{H}\)]nositol (20 Ci/mmol) was from Amersham-Buchler (Braunschweig, Germany).

Reagents, Antisera, and Hormones—Restriction enzymes and DNA-modifying enzymes (calf intestinal phosphatase, T4 DNA ligase, T4 polynucleotide kinase, DNase I), Moloney murine leukemia virus reverse transcriptase, and Tag DNA polymerase were from Strategene (Mannheim, Germany), New England Biolabs (Schwalbach, Germany), and Stratagene (Heidelberg, Germany). Sequenase was obtained from U.S. Biochemical (Bad Homburg, Germany). DNA molecular weight markers, agar, peptone, and yeast extract were purchased from Life Technologies (Eggenstein, Germany). Agarose (Sea-Kem and Sea-Plaque) was from Bioyam (Hessisch Oldendorf, Germany). Oligonucleotide primers were obtained from Tib Mol Biol (Berlin, Germany).

Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). A PLC-\(\beta\)-specific antisemur (Q-5) and blocking peptides were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Carbochol, forskolin, 3-isobutyl-1-methylxanthine, PGE\(_2\), and lysosphosphatic acid were from Sigma (Deisenhofen, Germany). Fura-2/AM was from Molecular Probes (MøbiTec, Gottingen, Germany), and hCG was from Calbiochem (Bad Soden, Germany).

Highly purified human pituitary LH (AFP-4261A) was donated by the NIDDK (National Institutes of Health, Bethesda, MD). Sources of additional materials have been described (16, 17).

**Methods**

Cell Culture—The development of LHR 11/6 cells stably expressing the murine LH receptor, LMS-40 cells stably expressing the rat \(M_2\) muscarinic, L\(\beta\)27 cells permanently expressing the human \(\beta_2\) adrenergic, and V2E11 cells expressing the human \(V_2\) vasopressin receptors have been described before (3, 13, 18). The cell lines were cultured at 5% CO\(_2\) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 \(\mu\)g/ml). Twenty-two out of 40 clones tested (55%) displayed a reduced hCG-

**Assessment of Intracellular Ca\(^{2+}\) Concentrations Using the Fluorescent Inducer Fura-2/AM—**Confluent cells were washed with PBS without Ca\(^{2+}\) and Mg\(^{2+}\) and subsequently incubated with PBS containing 5 \(\mu\)M EDTA at 37 °C for 5 min. Cells were detached by repeatedly flushing with growth medium and centrifuged at room temperature for 4 min at 100 × g, and the pellet was resuspended (2–2.5 × 10\(^5\) cells/ml) in 0.2 ml of cold buffer (IC buffer). 0.5 ml of IC buffer was added, and the solubilized membranes were centrifuged (12,000 × g for 10 min at 4 °C) to remove insoluble material. Antisera (15 \(\mu\)l) were added to the supernatants, and the samples were incubated for at least 2 h at 4 °C under constant rotation. Protein A-Sepharose beads (50 \(\mu\)l of 10% w/v) in precipitating buffer were added, and samples were further incubated at 4 °C overnight. Subsequent steps were performed as described in detail elsewhere (17).

**LH Receptor Signal Transduction**

LHR 11/6 cells were cotransfected with the \(\alpha\)-\(\beta\)-subunits was quantified by densitometric analysis of proteins separated by SDS-PAGE, processing of immunoblots, and detection into control and stimulated samples. Blotting of membrane proteins separated by SDS-PAGE, processing of immunoblots, and detection into control and stimulated samples. Blotting of membrane proteins separated by SDS-PAGE, processing of immunoblots, and detection into control and stimulated samples. Blotting of membrane proteins separated by SDS-PAGE, processing of immunoblots, and detection into control and stimulated samples. Blotting of membrane proteins separated by SDS-PAGE, processing of immunoblots, and detection into control and stimulated samples. Blotting of membrane proteins separated by SDS-PAGE, processing of immunoblots, and detection into control and stimulated samples. Blotting of membrane proteins separated by SDS-PAGE, processing of immunoblots, and detection into control and stimulated samples.
presence of 20 mM MnCl₂. Concentrations of cytosolic Ca²⁺ were calculated according to Gryncziewicz et al. (22).

Measurement of Inositol Phosphate Accumulation—Loading of cells with myo-[³H]inositol, stimulation with agonists, and Dowex chromatography have been described in detail elsewhere (13). The accumulation of inositol phosphates was normalized by dividing the counts reflecting [³H]inositol phosphates by the sum of counts for myo-[³H]inositol plus [³H]inositol phosphates. Results are expressed as percentage of inositol phosphates formed.

Determination of Intracellular cAMP Accumulation—Cells grown in six-well tissue culture dishes were washed 3 times with PBS and were preincubated for 15 min in 1 ml of assay buffer A (PBS supplemented with 1 mM CaCl₂, 1 mM MgCl₂, 0.2% glucose, 0.1% bovine serum albumin, and 1 mM 3-isobutyl-1-methylxanthine) at 37°C. Additives were then added, and the cells were incubated for 20 min at 37°C to allow cAMP accumulation to proceed. The reaction was stopped by placing the cells on ice and by aspiration of the incubation medium. Wells were then washed with ice-cold assay buffer B (identical to assay buffer A but not containing bovine serum albumin). In order to lyse the cells, 200 µl of ice-cold 3.9% trichloroacetic acid were added to wells containing 1 ml of assay buffer B. Samples were kept on ice for 10 min before the contents of the wells were transferred to Eppendorf tubes, vortexed, and centrifuged for 5 min at 12,000 × g. The supernatant (500 µl) was transferred to a fresh tube containing 370 µl of 1N KOH. The KClO₄ precipitate was pelleted, and the cAMP content of the supernatant was determined by radioimmunoassay (54).

Analysis of the mRNA Expression of Phospholipase C-β2 and -β3 Isoforms—The expression of PLC-β2 and -β3 isoforms in LHR 11/6 and HL 60 cells was studied by RT-PCR. Total RNA from cell lines was prepared as described in Zhu et al. (23), and poly(A)⁺ RNA was isolated as indicated in Guimard et al. (3). Reverse transcription and polymerase chain reaction were performed as described before (23) with minor modifications. Random hexanucleotides (50 pmol/reaction) were used to prime the reverse transcription reaction. A 1,068-bp fragment of PLC-β2 cDNA was amplified by PCR using sense primer P2.1, corresponding to nucleotide positions 1560 (24) plus EcoRI site and GC, and antisense primer P2.2, corresponding to nucleotide positions 1538–1512, plus EcoRI site and GC. PLC-β3 cDNA was amplified as a 1,292-bp fragment using sense primer P3.1, corresponding to nucleotide positions 1492–1512, plus XbaI site and GC and antisense primer P3.2, corresponding to nucleotide positions 2748–2767 (25), plus EcoRI site and GC. The reaction was incubated using a TRIO block DNA thermal cycler (Biometra, Göttingen, Germany) programmed to repeat the following times 25 times at 94°C for 1 min at 54°C, and 1.5 min at 72°C. The reaction mixture was resolved on a 1% agarose gel. Following standard protocols (26), specific fragments were cut out with XbaI, EcoRI and inserted into pBluescript SK⁺ that had been digested with the same restriction enzymes. Both inserts were sequenced by the dyeoxy chain termination method, applying the primer walk technique as described before (3). Sequence analysis was carried out using the MacVector software package (Softgen, Berlin, Germany).

Transfected of Phospholipase C-β2—Human PLC-β2 cDNA was excised from plasmid pVL1393-PLC-β2 by digestion with EcoRI/XbaI and inserted into the eukaryotic expression vector pcDNA3 (Invitrogen, Leek, The Netherlands), resulting in the construction of pcDNA-PLC. Cells were seeded into 12-well tissue culture plates (1 × 10⁵ cells/well) and grown to 50–60% confluency. On the day of transfection, culture media were removed, and 0.25 ml of Opti-MEM (Life Technologies) containing 6 µl of lipofectamine (Life Technologies) and the indicated amount of pcDNA-PLC-β2 plasmid DNA were added to each well. Five hours later, 0.25 ml of α-modified MEM were added, and the cells were labeled with myo-[³H]inositol (2 µCi/ml). Agonist-stimulated inositol phosphate accumulation was monitored 40–45 h after transfection as described elsewhere (13). To monitor PLC-β2 expression by immunoblotting, LHR 11/6 cells were seeded in 100-mm dishes, and the amount of PLC-β2 expressed was determined as described elsewhere (3). Cell lysates were added into LHR cells by lipofection using lipofectamine (Life Technologies) as recommended by the manufacturer. Harvesting of transiently transfected LHR 11/6 cells, preparation of cytosolic extracts, SDS-PAGE, and immunoblotting with an antisera reactive against PLC-β2 were performed as outlined in Ref. 27.

Miscellaneous Methods—Immunoblot analysis of β-ARK1 carboxyterminal expression was done according to Ref. 19. Binding of [³H]-PGE₂ to LHR cells was performed as described in Ref. 28. Data were analyzed using the Prism™ software package (Graph Pad Software, CA).

**RESULTS**

Immunoblotting of G Protein α-subunits Expressed in LHR Cells and Bovine Corpus Luteum Membranes—In order to characterize the G protein-coupling pattern of the LH receptor, two types of cell membranes were analyzed in the first set of experiments: membranes prepared from LHR cells, a clonal strain of mouse fibroblasts that have been stably transfected with a murine LH receptor cDNA (3), and bovine corpus luteum membranes endogenously expressing the LH receptor. To determine the G protein α-subunit expression in both systems, immunoblotting experiments with subtype-specific antipeptide antibodies raised against different α-subunits were performed (Fig. 1). An antisera raised against a C-terminal peptide sequence of α₂ (AS 348) recognized two proteins with an apparent molecular mass slightly exceeding 43 kDa in both membrane preparations (see Fig. 1, A and B). This relative molecular mass is close to that predicted from the amino acid sequences of the long and short splice variants of α₂ (see Ref. 29). It is noteworthy, however, that in luteal membranes both splice variants appear to be expressed to a similar extent (see Fig. 1B), whereas the low molecular weight band indicating the presence of the short isoform is hardly visible in L cell membranes (see Fig. 1A). An antibody specific for the G-protein...
α-subunits α₁ and α₁₁ (AS 370) showed the expression of corresponding 42-kDa proteins in both membranes. Under the separation conditions chosen (see “Experimental Procedures”) the upper band represents α₁₁, and the lower band represents α₃ (15). Immunoblotting of membranes with two antisera raised against the C-terminal sequence of α₁₁ (AS 233) and α₁₃ (AS 343) recognized two proteins of approximately 43 kDa. In addition, AS 233 cross-reacts with several unknown proteins of higher (see Fig. 1A) or lower molecular weights (see Fig. 1B).

An antibody recognizing all three α₁-subunits (α₁ common, AS 266) allowed us to visualize proteins of approximately 40 kDa in both membranes (see Fig. 1, C and D). By SDS-PAGE on 9% (w/v) acrylamide, 6 M urea gels, α₃ proteins can be resolved as two distinct bands, the upper band representing α₁ and α₃₁ and the lower band indicating the presence of α₁₂ (21). In order to differentiate between the expression of α₁1 and α₁₃ in L cells and bovine corpus luteum membranes, immunoblots were carried out using α₁₁ (AS 190)- and α₁₃ (AS 105)-specific antibodies. Fig. 1, C and D, shows that in both membrane preparations only AS 105 detected a specific 40-kDa band that migrated identically to the upper band visualized by the α₃ common antibody. Immunoblot experiments using an α₃ common antiserum (AS 6) antibody did not reveal specific bands in L cell and bovine corpus luteum membranes (see Fig. 1, C and D), although α₃ proteins were easily detectable in cholate extracts of bovine brain membranes using the same antibody (not shown).

Photolabeling of Receptor-activated G Proteins—To study coupling of the activated LH receptor to PTX-insensitive G proteins, LHR cell membranes were photolabeled with [α-32P]GTP aziadoanilide in the absence or presence of hCG at saturating concentrations (10 μg/ml, 220 nM). Photolabeled α₁-subunits were subsequently immunoprecipitated with the α₃ (AS 348), α₁₁ (AS 370), α₁₂ (AS 233), or α₁₃ (AS 343) antisera, and immunoprecipitates were resolved by SDS-PAGE. Activated G protein α₁-subunits were visualized by autoradiography (Fig. 2). Immunoprecipitation with the α₃ antisem revealed agonist-induced incorporation of [α-32P]GTP aziadoanilide into a 44- to 45-kDa protein corresponding to the long splice variant of α₃ (see Fig. 2A; range of [α-32P]GTP aziadoanilide incorporation into agonist-stimulated membranes compared with unstimulated controls, 4.1–5.3-fold, n = 5). In this and all further experiments preimmune antisera did not precipitate any photolabeled proteins, demonstrating that precipitation with the antisera employed in this study was specific. Stimulation of LHR cell membranes with hCG did not lead to increased incorporation of radioactivity into the α₁₁₁ or into the α₁₂- or α₁₃-subunits (Fig. 2A). Identical results were obtained by stimulation of membranes with highly purified LH (not shown).

In order to analyze whether agonist-induced activation of G₃ and G₁₃ could be monitored in L cells, membranes prepared from LM5-40 cells were studied (see Fig. 2B). LM5-40 cells are a donor strain of L cells that have been stably transfected with a rat M₁ muscarinic receptor (18), which couples to PLC-β via a PTX-insensitive G protein (30). In LM5 cell membranes carbachol (1 mM)-induced photolabeling of α₁₂ and α₃ could not be observed (see Fig. 2B). However, immunoprecipitation with the α₁₃ antisem resulted in agonist-dependent incorporation of [α-32P]GTP aziadoanilide into the 42- and 43-kDa α₁₁₁-subunits of G₁₃ (Fig. 2B; range of [α-32P]GTP aziadoanilide incorporation into agonist-stimulated membranes compared with unstimulated controls, 2.7–3.4-fold, n = 4). Thus, as opposed to the hCG-activated LHR receptor, G₁₃₁ activation elicited by the agonist-occupied M₁ muscarinic receptor can be monitored in the same parent cell line under identical experimental conditions. These findings indicate that the murine LH receptor expressed in L cells does not stimulate PLC via activation of G₁₃₁.

To test the hypothesis that the inability to demonstrate coupling of the LH receptor to G₁₃₁ proteins might be due to functional constraints inherent to the recombinant model system, the G protein-coupling pattern of the LH receptor was also studied in the receptor’s native environment, i.e., in membranes prepared from bovine corpora lutea. Davis et al. (31) demonstrated that LH increases inositol phosphates and cytosolic free Ca²⁺ in bovine luteal cells. In agreement with the results obtained with L cells, Fig. 3 shows that stimulation of membranes with 10 μg/ml (220 nM) hCG leads to an increased incorporation of [α-32P]GTP aziadoanilide into α₃ (range of [α-32P]GTP aziadoanilide incorporation into agonist-stimulated membranes compared with unstimulated controls, 3.5–4.6-fold, n = 4) but not into α₁₁₁, α₁₂, and α₁₃.
PTX-sensitive G proteins. Gi2 and Gi3 have been identified as radioactivity into the lower molecular weight bands of hCG (220 nM) and immunoprecipitated with the α-antiserum, as described. Immunoprecipitated proteins were subjected to SDS-PAGE. Numbers on the left of the figure represent molecular masses of standard proteins. Results from one of four independent experiments are shown.

Next we decided to investigate coupling of the LH receptor to PTX-sensitive G proteins. Gi2 and Gi3 have been identified as PTX-sensitive G proteins expressed in L cells and bovine corpora lutea (see Fig. 1, C and D). Following hCG stimulation (10 μg/ml, 220 nM) in both systems, immunoprecipitation with an α-antiserum (AS 266) revealed increased incorporation of radioactivity into the lower molecular weight bands of ~40 kDa (Fig. 4A; range of α-[32P]GTP azidoanilide incorporation into agonist-stimulated membranes compared with unstimulated controls, 1.8–2.6-fold, n = 4; Fig. 4B, 3.1–3.6-fold, n = 4). Thus, we were able to demonstrate that in addition to Gs, the agonist-activated LH receptor couples specifically to Gi2 in L cells and in bovine corpora lutea.

Functional Coupling of the LH Receptor to Gs—To examine the functional significance of coupling of the LH receptor to Gi proteins, the effect of PTX treatment of LHR cells on agonist-stimulated cAMP accumulation was tested. LHR cells were treated with 100 ng/ml of PTX 3 times at 12-h intervals beginning 36 h prior to assay (30). Subsequently, the cells were washed with PBS and incubated with 0.1% bovine serum albumin (basal), hCG (1 nM), PGE1 (10 μM), or forskolin (FSk, 10 μM) as indicated. After an incubation period of 20 min, cells were washed and lysed as described under Experimental Procedures, and intracellular cAMP accumulation was determined by an in-house radioimmunoassay. Data (mean ± S.D.) are from one of four representative experiments, each performed in triplicate.

In a second set of experiments the ability of the LH receptor to inhibit cAMP formation was studied. LHR cells were treated with 10 μg/ml of CTX for 24 h and subsequently stimulated with hCG at increasing concentrations (0–10 nM) in the presence of 10 μM forskolin. Forskolin was added to secure agonist-independent activation of the remainder of Gs that had not been ADP-ribosylated during toxin pretreatment. As shown in Fig. 6, cells that had not been treated with CTX responded to hCG with a concentration-dependent increase in cAMP accumulation. On the contrary, CTX treatment precluded a further agonist-induced stimulation but resulted in a concentration-dependent inhibition of cAMP accumulation (see Fig. 6). Taken together, these results demonstrate functional coupling of the LH receptor to Gi proteins belonging to the Gi family.

Involvement of Gs in LH/hCG-dependent Phospholipase C
Ca\(^{2+}\) by 100 \(\mu\)m carbachol was also unaffected by prior treatment of cells with pertussis toxin (not shown). These observations provide functional evidence for different mechanisms being operative in the mobilization of Ca\(^{2+}\) brought about by the activated LH as compared with the M\(_5\) muscarinic and the endogenous P\(_2\) purinergic receptors in L cells.

In order to further analyze the involvement of G proteins in the stimulation of PLC activity by hCG, LHR and LM5 cells were pretreated with PTX (see above), and inositol phosphate formation was monitored in response to several agonists (Fig. 7). As illustrated in Fig. 7A, the addition of 220 nm hCG to LHR cells prelabeled with myo-[\(^{3}\)H]inositol resulted in a stimulation of inositol phosphate production that was markedly diminished if cells had been pretreated with PTX. It should be noted, though, that even the extensive PTX treatment scheme applied in this study did not result in complete abolition of hCG-induced inositol phosphate formation. In fibroblasts and various other cell types, the phospholipid lysophosphatidic acid promotes cell proliferation by signaling through a PTX-sensitive G protein that is believed to be G\(_i\) (32). Stimulation of LHR cells with 10 \(\mu\)M lysophosphatidic acid led to substantial stimulation of inositol phosphate production, which could largely, if not completely, be suppressed by PTX pretreatment (see Fig. 7B). These findings in LHR cells demonstrate that PLC activity can be stimulated via PTX-sensitive G proteins in L cells. In contrast to the results obtained in LHR cells, activation of the M\(_5\) muscarinic receptor in LM5 cells resulted in an increased inositol phosphate formation that was unaffected by PTX (see Fig. 7C), reflecting coupling of the M\(_5\) muscarinic receptor to G\(_{\beta\gamma}\) (see Fig. 28).

Involvement of G-protein \(\beta\gamma\)-Subunits in LH/hCG-dependent Phospholipase C Activation—Assuming that PLC activation by G proteins is mediated by \(\beta\gamma\)-subunits released from the activated heterotrimer (10), one would have to postulate that \(\beta\gamma\)-sensitive isoforms of PLC-\(\beta\) are expressed in L cells. To address this issue, we isolated poly(A)\(^+\) RNA from LHR and also from HL 60 cells and analyzed the RNA by RT-PCR for the expression of phospholipases C-\(\beta\)2 and -\(\beta\)3 mRNAs. Fig. 8 shows that DNA fragments of the expected length for the \(\beta\)2 (1068 bp) and \(\beta\)3 (1292 bp) isoforms could be amplified from either template. The identity of the bands was confirmed by subcloning and DNA sequencing. The 500-bp fragment that is additionally obtained when using PLC-\(\beta\)2-specific primers reflects unspecific annealing.

If the LH receptor is able to activate \(\beta\gamma\)-stimulable isoforms of PLC-\(\beta\), overexpression of PLC-\(\beta\)2 in LHR cells should increase hCG-dependent phosphatidylinositol hydrolysis. On the contrary, PLC-\(\beta\)2 overexpression in LM5 cells should not affect carbachol-induced inositol phosphate accumulation as the M\(_5\) muscarinic receptor is coupled to G\(_{\gamma}\). As illustrated in Fig. 9A, transient transfection of increasing amounts of cDNA coding for PLC-\(\beta\)2 into LHR cells resulted in increased concentration-dependent increase in hCG (220 nm)-stimulated inositol phosphate accumulation, whereas overexpression of PLC-\(\beta\)2 in LM5 cells had no effect on carbachol (100 \(\mu\)M)-dependent inositol phosphate formation (Fig. 9B). In order to independently demonstrate that transient transfection of LHR cells with increasing amounts of PLC-\(\beta\)2 cDNA resulted in increased expression of PLC-\(\beta\)2, immunochemical analyses were performed (Fig. 9C). Extracts of cells were subjected to SDS-PAGE, and immunoblotting using an antiserum reactive against PLC-\(\beta\)2 specifically detected a protein of approximately 165 kDa if cells had been transiently transfected with PLC-\(\beta\)2 cDNA. The magnitude of PLC-\(\beta\)2 expression positively correlated with the amount of cDNA used for transfection (see Fig. 9C).

As PTX treatment of LHR cells did not completely abolish hCG-induced PLC activation (see Fig. 7A), \(\beta\gamma\)-subunits released from G\(_i\) may play a role in the cells’ response. If this assumption holds true, activation of other G\(_s\)-coupled receptors expressed in L cells should also result in PLC-\(\beta\) stimulation. Challenging LHR 11/6 cells with 10 \(\mu\)M PGE\(_1\) (1500 prostaglandin binding sites/cell), however, did not lead to agonist-induced inositol phosphate accumulation (Fig. 10A). On the contrary, L cell lines permanently expressing high numbers of \(\beta\)2-adrenergic (L\(_\beta\)2/7 cells: 300,000 sites/cell) or \(\beta\)2 vasopressin receptors (V2E11: 500,000 sites/cell) responded to the respective agonists with increased PLC activity that was unaffected by PTX treatment (Fig. 10, B and C). Agonist-induced mobilization of Ca\(^{2+}\) from intracellular stores has additionally been shown for L\(_\beta\)2/7 and V2E11 cells (13). Considering that the latter two receptors do not appear to couple to G\(_{\gamma}\) (33, 34), a participatory role of \(\beta\gamma\)-subunits released from G\(_i\) in agonist-induced PLC stimulation can be inferred.

If \(\beta\gamma\)-subunits released from G\(_i\) and G\(_s\) contribute to hCG-stimulated PLC activity in LHR cells, removal of free \(\beta\gamma\)-subunits should reduce hCG-induced PLC activity more effectively than PTX treatment. To address this issue, the \(\beta\gamma\)-binding C-terminal \(\beta\gamma\)-ARK1 polypeptide (19) was stably expressed in LHR 11/6 cells, and the expression of the peptide in different cell lines was demonstrated by immunoblotting (Fig. 11A). Expression of the \(\beta\gamma\)-ARK1 polypeptide led to a marked reduction (LHR-\(\beta\gamma\)-ARK 36) or complete abolition (LHR-\(\beta\gamma\)-ARK 8) of hCG-stimulable PLC activity (Fig. 11B), whereas hCG-dependent adenyl cyclase activation was clearly measurable with only minor variations in EC\(_{50}\) values between different cell lines (data not shown). In summary, these data strongly support the notion that \(\beta\gamma\)-subunits released from G\(_i\) and G\(_s\) are involved in dual coupling of the LH receptor to adenylyl cyclase and PLC-\(\beta\) isoforms.

**DISCUSSION**

There is a large body of evidence indicating that binding of LH or hCG to its cognate G-protein-coupled receptor sets in motion at least two signaling cascades involving CAMP-dependent protein kinase and protein kinase C (1). Activation of adenylyl cyclase and PLC have been identified as early events upstream in the LH/hCG-initiated signaling cascade (3–5). The mechanism that governs this dual signaling is not clear at present. We therefore set out to identify the G proteins that participate in this signaling (LHR-

| Additions          | Control | PTX-treated cells |
|--------------------|---------|-------------------|
| None               | 69 ± 6  | 60 ± 4            |
| hCG, 10 \(\mu\)g/ml (220 nm) | 149 ± 20 | 81 ± 6           |
| ATP, 100 \(\mu\)M | 216 ± 23 | 205 ± 11         |

**TABLE I**

Effect of pertussis toxin treatment on agonist-induced Ca\(^{2+}\) mobilization in LHR 11/6 cells

Cells were grown as monolayers to confluence, washed with PBS, and loaded with the fluorescent dye Fura-2/AM as described under “Experimental Procedures.” In aliquots of loaded cells, concentrations of intracellular Ca\(^{2+}\) were monitored in the absence of agonists, or agonist-induced peak concentrations were determined in the presence of hCG or ATP. Control cells were compared with those that had received pertussis toxin treatment (0.1 \(\mu\)g/ml, 3 times at 12-h intervals beginning 36 h prior to harvesting). Results represent basal or peak cellular Ca\(^{2+}\) concentrations (\(\mu\)M) from four independent experiments (mean ± S.D.).
G₁₃, G₂α, and G₃α, whereas G₀ expression could not be demonstrated. The experimental approach that was chosen to monitor receptor-G protein interaction is a combination of receptor-dependent photolabeling of G protein α-subunits with subsequent immunoprecipitation. This approach has been reliably applied to detect receptor-G protein interactions in a variety of systems (21, 35, 36). Photolabeling experiments employing the nonhydrolyzable GTP analog [α-³²P]GTP azidoanilide showed that in LHR cells the activated LH receptor couples to G₂ and to G₁ proteins. The inability to detect coupling of the LH receptor to G₁₃ and G₀ was due to the recombinant system, as in the same parent cell line carbachol-induced activation of G₀ and G₁₃ could clearly be demonstrated under identical experimental conditions. Furthermore, expression levels of LHR receptors in LHR 11/6 cells (24,000 receptors/cell; see Ref. 3) and M₅ muscarinic receptors in LMS-40 cells (75,000 receptors/cell; see Ref. 18) are comparable; and, pursuing a photolabeling approach, it has been shown that the ability of the β₂-opioid receptor to interact with multiple G proteins is independent of receptor density even if receptor numbers per cell vary by nearly 2 orders of magnitude (36). In order to rule out the possibility that certain receptor-G protein coupling patterns would not occur in recombinant cell lines as opposed to cells endogenously expressing the receptor of interest, we also analyzed LH receptor-G protein coupling in bovine corpus luteum steroidogenic cells. In both cell systems studied herein, the G₀ protein activated by the LH receptor can be specified as G₂α.

An early event following LH receptor activation in gonadal steroidogenic cells is G₃-mediated elevation of intracellular cAMP levels (1). Thus, coupling of the activated LH receptor to G₀ proteins may be looked upon as being confusingly counter-productive because activation of a G₁-dependent signaling pathway.
Coupling of the LH receptor to Gαi, which usually represents a major portion of cellular heterotrimeric G proteins, may be functionally relevant with respect to PLC activation. In keeping with this notion we observed that PTX treatment of LHR cells impaired hCG-induced Ca²⁺ mobilization and inositol phosphate accumulation. However, even if a stringent treatment schedule is applied, a PTX-insensitive component remains. L cells express PLC isozymes β2 and β3, which are potential targets for βγ-subunits released from activated Gα (10). The fact that overexpression of PLC-β2 in LHR cells results in augmented hCG-induced inositol phosphate accumulation, whereas carbachol-dependent inositol phosphate formation in LM5 cells is unaffected, provides a strong argument in favor of the involvement of βγ-subunits in hCG-dependent PLC activation. It has been shown that the concentration of βγ-subunits required for half-maximal activation of PLC-β2 and -β3 is considerably higher than that for αβ on PLC-β1 (41). Therefore, βγ-mediated stimulation of PLC may require a higher incidence of receptor-G protein collisions than necessary for the stimulation of adenylyl cyclase by αβ. This could explain the observed rightward shift of the concentration-response curve for PLC activation as compared with that for adenylyl cyclase activation (3).

The nature of the PTX-insensitive component contributing to hCG-stimulated PLC activity in LHR 11/6 cells requires careful consideration. We cannot rule out with absolute certainty that there might be an additional, rather inefficient, LH receptor-Gαi interaction that was not detected for reasons of assay sensitivity. However, our data strongly support a model in which βγ-subunits released from activated Gα may be a major portion of cellular heterotrimeric G proteins, may be functionally relevant with respect to PLC activation. In keeping with this notion we observed that PTX treatment of LHR cells impaired hCG-induced Ca²⁺ mobilization and inositol phosphate accumulation. However, even if a stringent treatment schedule is applied, a PTX-insensitive component remains. L cells express PLC isozymes β2 and β3, which are potential targets for βγ-subunits released from activated Gα (10). The fact that overexpression of PLC-β2 in LHR cells results in augmented hCG-induced inositol phosphate accumulation, whereas carbachol-dependent inositol phosphate formation in LM5 cells is unaffected, provides a strong argument in favor of the involvement of βγ-subunits in hCG-dependent PLC activation. It has been shown that the concentration of βγ-subunits required for half-maximal activation of PLC-β2 and -β3 is considerably higher than that for αβ on PLC-β1 (41). Therefore, βγ-mediated stimulation of PLC may require a higher incidence of receptor-G protein collisions than necessary for the stimulation of adenylyl cyclase by αβ. This could explain the observed rightward shift of the concentration-response curve for PLC activation as compared with that for adenylyl cyclase activation (3).

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vation of mitogen-activated protein kinase in COS-7 cells (46).

The LH, follicle-stimulating hormone, and thyroid-stimulating hormone receptors form the subfamily of glycoprotein hormone receptors. These receptors share common structural features and display a considerable degree of sequence homology, particularly within the transmembrane domains (3). Thus, it is surprising to realize that the LH and the thyroid-stimulating hormone receptors differ in their G protein-coupling pattern. As opposed to the LH receptor coupling to G_s and G_i, the thyroid-stimulating hormone receptor has been reported to couple to G_s and G_{null} (15). It will be interesting to identify the molecular basis for such functional differences of two closely related receptors and to describe how the follicle-stimulating hormone receptor relates to this scenario.

The physiologic importance of the ability of the LH receptor to activate at least two signaling cascades is not clear. The ability of the activated LH receptor expressed in L cells or HEK 293 cells to stimulate inositol phosphate accumulation is dependent on a high hormone concentration (3) and on receptor density. A minimal threshold density has to be reached in order for hCG-stimulated PLC activation to be measured. In L cells this threshold lies between 500 and 4,000 LH receptors/cell (13) and between 1 and 2 × 10^5 receptors in HEK 293 cells (5, 47, 48). One of the critical steps during follicular growth is the induction of LH receptors in granulosa cells, a process that is mediated by the concerted actions of both follicle-stimulating hormone and estradiol (2, 49). It is therefore tempting to speculate that at the time of ovulation, LH concentrations and LH mitogenic effects of LH remains unknown. Our demonstration of Gi activation by the LH receptor expressed in L cells should be enlightening in the process of ovulation, e.g. proestraglandin synthase-2 (summarized in Ref. 2).

βγ-Subunits released from activated heterotrimeric G proteins, mainly from G_s, regulate the activity of enzymes like adenyl cyclase and PLC-β isomers (10, 50) or phosphatidylinositol 3-kinase (51) but have recently also been shown to be involved in the regulation of mitogen-activated protein kinases (summarized in Ref. 52). LH/hCG-induced ERK activation was demonstrated in COS-7 cells transiently transfected with the cDNA coding for the LH receptor (53). The effect could partly be mimicked by CAMP, but the precise mechanism underlying the mitogenic effects of LH remains unknown. Our demonstration of G_s activation by the LH receptor should be enlightening in this regard.

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