The luteinizing hormone/chorionic gonadotropin receptor has distinct transmembrane conductors for cAMP and inositol phosphate signals.*

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The luteinizing hormone/chorionic gonadotropin receptor is a member of the seven-transmembrane receptor family. It is coupled, presumably via Gα and Gq, to two signal pathways involving adenylyl cyclase/cAMP and phospholipase C/inositol phosphate (IP). Little is known about the events prior to G-protein coupling: for example, whether these signals are generated from a single or multiple independent origins and mechanisms, when and where they diverge, and how they are transduced. We report novel observations that the cAMP signal and the IP signal originate and diverge upstream of G-protein coupling. The generation of these two signals independently involves Lys583 in exoloop 3 of the rat receptor. For this study, Lys583 of the receptor was substituted with a panel of amino acids, and mutant receptors were assayed for hormone binding and induction of cAMP, inositol monophosphate, inositol bisphosphate, and inositol trisphosphate. No substitutions for Lys583 were permissible for cAMP induction, despite successful surface expression and hormone binding. In contrast, several substitutions were permissible for IP induction. Our results suggest two distinct transmembrane signal conductors for cAMP and inositol phosphate signals and imply particular models of receptor activation not previously suggested.

The LH/CG receptor is a member of the seven-transmembrane (TM) receptor family and comprises two distinct functional domains consisting of the extracellular N-terminal half and the membrane-associated C-terminal half. These two domains are encoded by 11 exons (1, 2). Exons 1–10 encode the extracellular N-terminal domain, which alone is responsible for high affinity hormone binding (3–5). This distinguishes the LH/CG receptor and other glycoprotein hormone receptors from all other seven-TM receptors, which have the high affinity ligand binding sites in the membrane-associated domain (6). Exon 11 of the LH/CG receptor encodes the membrane-associated domain, which is capable of low affinity hormone binding and receptor activation (5, 7). This membrane-associated domain includes seven-TM helices joined together by three exoloops and three cytoloops.

The LH/CG receptor is coupled to the adenylyl cyclase and PLC signal pathways, inducing the production of cAMP and inositol phosphates (IPs) as intracellular signal molecules (8). These signal molecules are produced when the receptor couples, presumably through Gα and Gq, to adenylyl cyclase and PLC, respectively (9). However, little is known about the events prior to G-protein coupling (6) (in particular, when more than one signal is involved). As a first step to lead discussion, several simple mechanisms are hypothesized for dual signals as shown in Fig. 1. Hormonal signal generation and transfer can be likened to an electrical circuit. Contact between the hormone and the receptor acts as an on/off signal switch and the receptor conducts the signal to the terminal cytodomain G-protein dock. Signals may be generated and transferred by single switch/single conductor, single switch/dual conductors, or dual switches/dual conductors. We will examine these models in light of the existing evidence.

Recent investigations have identified several amino acids of the LH/CG receptor that are important for receptor activation (11–19). Most of these amino acids are present in cytoplasmic or TM domains. Only a few were found in the extracellular regions of the LH/CG receptor (11, 12, 17–19). The added significance of extracellular amino acids in receptor activation is that they are near or at the hormone binding domain and could be involved in signal generation (6). Although their involvement in cAMP induction has been extensively examined, none of these extracellular amino acids were examined for their role in the PLC pathway and IP induction. The existing data are not useful for studying the mechanisms of dual signals and the models in Fig. 1. Therefore, it became necessary to reevaluate the activation of the LH/CG receptor and to rigorously determine IP induction.

Recently, we reported that Lys583 of exoloop 3 was uniquely essential for cAMP induction, whereas all other amino acids of exoloop 3 were not (19). Lys583 is located at the boundary of exoloop 3 and TM helix 7. Therefore, the amino acid at this position is expected to play a crucial role in maintaining the structure and orientation of the TM 7 helix, thus influencing its function (6). In fact, the substitution for Lys583 of a variety of amino acids resulted in the loss of cAMP induction, regardless of the charge, length, shape, and hydrophobicity of the side chain, as did the deletion of Lys583 (19). However, little is known about the role of Lys583 in the PLC pathway and IP induction. In this communication, cAMP, total IP (IP3), inositol monophosphate (IP1), inositol bisphosphate (IP2), and inositol trisphosphate (IP3) were analyzed together. The results indicate that Lys583 independently affects IP and cAMP induction and is important for but not essential to IP induction. Our results suggest intriguing and differential roles of the Lys583 side chain in induction of cAMP, IP1, IP2, and IP3. Furthermore, a model of receptor activation is proposed, which defines two separate and distinct signal conductors.
Experimental Procedures

Mutagenesis and Functional Expression of LH/CG Receptors—Mutagenesis and functional expression of rat LH/CG receptors were carried out as described previously (12). Briefly, wild-type and mutant LH/CG cDNAs were prepared in pSELA vector using the altered site mutagenesis system (Promega) then subcloned into pcDNA3 (Invitrogen). Constructs were transfected into human embryonic kidney 293 cells, and stable cell lines were established in the presence of 500 μg/ml G-418 and grown at 37°C with 5% CO2. Stable cell lines were used to determine CG binding and induction of cAMP and IP. The CG batch CR 127 was provided by the National Hormone and Pituitary Program and used for all experiments.

cAMP Assay—cAMP was assayed for as described previously (19). Briefly, stable cell lines were incubated with increasing concentrations of CG in the presence of isobutylmethylxanthine (0.1 mg/ml) for 45 min at 37°C. The reaction was stopped by removing medium, washing cells once with medium, and lysing cells in 70% ethanol by freeze-thawing in liquid nitrogen. Cells were scraped from plate wells and centrifuged at 16,000 × g for 10 min at 4°C. The supernatant was collected, dried under vacuum, and assayed for total cAMP with a 125I-cAMP assay kit (Amersham Corp.) according to the manufacturer’s instructions.

Inositol Phosphate Assay—Stable cell lines were plated in 6-well plates, incubated with 2 μCi/ml [3H]inositol (DuPont, NEN), and grown in minimum Eagle’s medium plus 8% heat-inactivated horse serum for 48 h to 50–60% confluence. Wells were washed once with minimum Eagle’s medium without heat-inactivated horse serum and incubated in the same medium for 1 h at 37°C. The medium was removed and cells were washed once with phosphate-buffered saline supplemented with 5.5 mM glucose, 0.5 mM CaCl2, and 0.5 mM MgCl2, and incubated for 20 min at 37°C. Phosphate-buffered saline was removed and cells were incubated in phosphate-buffered saline containing 10 mM LiCl for 10 min at 37°C. After cells were stimulated for 30 min with increasing concentrations of CG, the stimulation was stopped by replacing phosphate-buffered saline with a mixture of chloroform/methanol/HCI (1:2:0.25, v/v/v). Cells were scraped and treated in a mixture of equal volumes of chloroform and water (20). Samples were briefly vortexed and centrifuged at 1000 × g for 5 min. The aqueous layer, which contained IP and was free of phospholipids, was recovered and dried on a vacuum concentrator. Dried samples were redissolved in 500 μl of 50 mM Tris-HCl and applied to Dowex AG-1 formate (Bio-Rad) columns. Each column was sequentially washed with 10 ml of water to remove unbound materials and 10 ml of 60 mM ammonium formate and 5 mM sodium tetraborate to elute free inositol and glycerol phosphoinositol. IP species were sequentially eluted with 10 ml each of 0.1 M formic acid in 0.2, 0.7, and 1.05 mM ammonium formate. Fractions of 1 ml were collected, and 200 μl was counted for radioactivity in 3 ml liquid scintillation mixture. Peak radioactivities were used for the data analysis.

Data Analysis—Binding and cAMP assays were performed in duplicate and repeated twice. IP assays were repeated 3–4 times. Mean and standard deviations were calculated and one-way analysis of variance was used to determine significant differences (p < 0.05) between values of wild-type and mutant LH/CG receptors.

Results

Hormone Binding and cAMP Induction—Previously, we have shown that mutant receptors with substitution of Ala, Arg, and Glu and deletion at the Lys(K583L, LH/CG-RK583E, LH/CG-RK583A, and LH/CG-RK583D, respectively) were expressed on the surface of 293 cells, whereas surface expression of other substitution mutant receptors was not detectable (19). The mutant receptors bound 125I-CG with Kd values similar to that of the wild-type receptor (p < 0.05). However, LH/CG-RK583L bound 125I-CG after solubilization in Triton X-100 but not on intact cells, indicating that most of the LH/CG-RK583L was trapped within the cells (19). Therefore, the cells were used as a negative control to determine surface expression.

cAMP Induction—The wild-type receptor induced cAMP with an EC50 value of 54 pM and a maximum cAMP level of 88 fmol/1000 cells (Fig. 2). None of the mutants, however, produced significant amounts of cAMP (p < 0.05). These results indicate that the Lys(K583L substitution lost the ability to activate the adenyl cyclase signal pathway. This loss of cAMP induction is specific for Lys(K583L since the A1a substitution for Lys573 at the junction of exoloop 3 and TM 6 did not have an impact on cAMP induction (16).

IPt and IP1 Induction—Phosphatidylinositol-specific PLC hydrolyzes various phosphatidylinositol phosphates to produce several inositol phosphates, IP1, IP2, and IP3 (21). Initially, we determined the intracellular concentration of IPt. The IPt concentration of the wild-type receptor sample increased in response to increasing CG concentrations, reaching a 1.8-fold apparent maximum increase at 1 μM CG. The EC50 value for IPt induction was 5.3 nM. To determine the exact composition of IPt induced by CG, individual inositol phosphates were analyzed. IP1 made up the majority of total IPt, and, therefore, IP1 was the most sensitive indicator of CG-dependent IPt induction. This predominant accumulation of IP1 may be the result of the hydrolysis of phosphatidylinositol by PLC or a LiCl-insensitive inositol phosphatase, which could convert IP3 into IP1 (21). Although our approach is not sensitive enough to distinguish between these possibilities, the CG-dependent IP1 accumulation demonstrates PLC activation. Lys(K583L substitution mutants produced disparate IP1 responses (Fig. 3). LH/CG-RK583E and LH/CG-RK583B did not produce IP1 (p < 0.05) in response to increasing concentrations of CG. This result is similar to no CG-dependent cAMP induction by the mutant receptors (Fig. 2). IP1 production by LH/CG-RK583A was marginally noticeable. Surprisingly, however, both LH/CG-RK583A and LH/CG-RK583D demonstrated CG-dependent IP1 induction. The maximum amount and –fold increase of IP1 induction by LH/CG-RK583D were similar to those of the wild-type receptor (p < 0.05). However, the EC50 value of LH/CG-RK583D was lower than that of the wild-type receptor. These data indicate that LH/CG-RK583A is capable not only of inducing CG-dependent IP1 production but of inducing it with a better affinity. This is strikingly different from its impotent cAMP induction. LH/CG-RK583D also responded to CG and the IP1 concentration increased by 1.4-fold with an EC50 value of 29 pM. The maximum amount of IP1 was relatively low, 25–30% of that produced by the wild-type receptor. This low maximum level of IP1 may be due to the low receptor concentration of LH/CG-RK583D. Interestingly, the less responsive and unresponsive mutant receptors showed low basal levels compared to the responsive receptors.

The affinities and maximum levels of IP1 induction by the receptors were similar to those of IP1. The wild-type receptor, LH/CG-RK583A, and LH/CG-RK583D responded positively to
whereas LH/CG-RK583E and LH/CG-RK583R were unresponsive. The basal IP1 levels of the mutant receptors were diverse and below that of the wild-type receptor, except for LH/CG-RK583A. Its basal level was similar to that of the wild type.

**IP2 and IP3 Induction—** The concentrations of IP2 and IP3 produced by all of the receptors were relatively low compared with those of IP1 (Fig. 4). One exception was LH/CG-RK583A, which responded to CG to produce IP2 and IP3 with EC50 values of 140 pM and 100 pM, respectively. The IP2 and IP3 levels peaked at 1 nM CG. Another notable feature is that the basal IP2 and IP3 levels of LH/CG-RK583Del were higher than those of the wild-type and other mutant receptors. It remains to be seen whether our observations of the changes in the IP basal levels reflect constitutive changes.

It is possible that mutant constructs undergo unintended changes in the DNA sequence. Therefore, it is necessary to verify that the amino acid and DNA sequences of all mutant receptors were exactly the same as that of the wild-type receptor except for the intended mutations. For this purpose all mutant constructs were reverted to the wild type by reverse mutagenesis and the resulting revertant receptors were examined. All of them regained the characteristics of the wild-type receptor in hormone binding, cAMP induction, and IP induction. Because the data are virtually identical to those of the wild-type receptor, they are not presented.

**DISCUSSION**

Our data indicate that mutant receptors, LH/CG-RK583A and LH/CG-RK583Del, were capable of inducing IP but not cAMP, whereas LH/CG-RK583R and LH/CG-RK583E were incapable of inducing either IP or cAMP. These effects are specific to the LH/CG receptor because the presence of CG and the surface expression of functional LH/CG receptors are required. In addition, mock-transfected cells were not responsive, whereas mutations of the LH/CG receptor had an impact on the IP induction. These results taken together with their successful surface expression and high affinity hormone binding indicate the important and independent roles of Lys583 and its side chain in the generation and conductance of IP and cAMP signals. The surface location of Lys583 suggests that both signals are generated at the cell surface, presumably near or at the hormone binding site. Our results also indicate the receptor domain for signal divergence located at or near Lys583 because the IP signal pathway remained intact while the cAMP signal was shorted. This result suggests the existence of distinct transmembrane signal conductors for cAMP and IP signals, as depicted in models C and D (Fig. 1). These signal conductors lead to the Gs and Gi terminals, respectively (9, 22, 23). It is not clear, however, whether the IP and cAMP signals require one switch or two independent switches. In the latter case, the hormone is capable of independently turning on the two

**FIG. 2.** Hormone binding and cAMP induction. Lys583 was substituted with Arg (positive charge), Ala (hydrophobic side chain), Leu (extended hydrophobic side chain), or Glu (negative charge) to produce LH/CG-RK583R, LH/CG-RK583A, LH/CG-RK583L, or LH/CG-RK583E. In LH/CG-RK583Del, Lys583 was deleted to avoid the effect of a newly introduced side chain. These mutant receptors were individually expressed on 293 cells and assayed for hormone binding and CG-dependent cAMP induction. For hormone binding, cells were incubated with a constant amount of 125I-CG and increasing concentrations of noniodinated CG as described. For cAMP assay, cells were incubated with increasing concentrations of CG and intracellular cAMP was determined. NS, not significant.

**FIG. 3.** Induction of IP1 and IP2. The mutant receptors described in Fig. 2 were assayed for IP1 and IP2 as described under “Experimental Procedures.” The resulting cpm value was analyzed as described in the Fig. 2 legend and presented either directly, as cpm, or converted to the fold increase over the basal level cpm of individual mutants. Background was 20 cpm and was <4% of peak IP counts.

**FIG. 4.** IP2 and IP3 induction. The concentrations of IP2 and IP3 produced by all of the receptors were relatively low compared with those of IP1 and IP2 (Fig. 4). One exception was LH/CG-RK583A, which responded to CG to produce IP2 and IP3 with EC50 values of 140 pM and 100 pM, respectively. The IP2 and IP3 levels peaked at 1 nM CG. Another notable feature is that the basal IP2 and IP3 levels of LH/CG-RK583Del were higher than those of the wild-type and other mutant receptors. It remains to be seen whether our observations of the changes in the IP basal levels reflect constitutive changes.

It is possible that mutant constructs undergo unintended changes in the DNA sequence. Therefore, it is necessary to verify that the amino acid and DNA sequences of all mutant receptors were exactly the same as those of wild-type receptor except for the intended mutations. For this purpose all mutant constructs were reverted to the wild type by reverse mutagenesis and the resulting revertant receptors were examined. All of them regained the characteristics of the wild-type receptor in hormone binding, cAMP induction, and IP induction. Because the data are virtually identical to those of the wild-type receptor, they are not presented.
Switches. On the other hand, the differential effect of Lys583 mutations on cAMP and IP induction is not consistent with model A (single switch/single conductor/single terminal) or model B (single switch/single conductor/dual terminals). When the side chain including the e-amine of Lys583 was replaced with side chains of other amino acids containing a positive charge, a negative charge, or a hydrophobic group, the cAMP signal was not generated. The position of Lys583 wedged between an extracellular domain and a TM helix, is not expected to readily be altered by the substitution with Arg and Glu. Furthermore, the charge of the side chain alone is not likely to be a sufficient factor for cAMP induction because the Arg substitution was not permissible. Therefore, other factors, including the geometry, physical shape, flexibility, hydrophobicity, and/or, perhaps, the charge, may be necessary for the structural requirement for cAMP induction. Because Lys583 is located at the junction of exoloop 3 and the TM 7 helix, substitution at or deletion of this amino acid is expected to affect the conformation and function of TM 7 (6). It remains to be seen how changes in these factors affected the interactions of Lys583 with other groups of the receptor and/or the hormone, thus influencing the structure of exoloop 3 and TM 7. One intriguing possibility is that Lys583 may ion-pair with Asp587 (12) at the junction of exoloop 1 and TM 2. When the seven TMs are arranged to form a TM tunnel, Lys583 of TM 7 may be near Asp587. Interestingly, these two counter-ions are important for cAMP induction (12). On the other hand, Lys573 and Asp587 might not form an ion pair as they are likely to be further apart in the TM tunnel.

In contrast to the strict structural requirement of Lys583 for cAMP induction and the futile cAMP signal generation upon the substitutions, the IP signal was not only successfully generated after the Lys583 → Ala substitution but also the affinity for IP induction was significantly improved by 43-74-fold. This affinity was similarly improved when Lys583 was deleted, suggesting that the amino group and its charge of Lys583 are inhibitory to and/or unnecessary for the IP signal generation. It is unlikely that removal of the positive charge, as in the LH/CGRK583 and LH/CGRK583D4 mutants, produces a receptor conformation more conducive to Go coupling. This is because other substitution mutants, particularly LH/CGRK583D4, were incapable of IP and cAMP induction despite successful surface expression. A more logical conclusion is that the signal for cAMP induction is shorted at the cell surface, whereas the IP signal remains active and travels its normal or more efficient conductance pathway.

Substitution mutations for other amino acids of the LH/CG receptor were shown to be responsible for elevated basal cAMP levels while maintaining the CG-dependent cAMP inducibility and the affinity for cAMP induction. They include Met571 → Ile (13), Ala572 → Val (14, 16), Thr577 → Ile (13, 16), Asp578 → Gly (24), and Asp578 → Tyr (16). Among these substitutions, Met571 → Ile (13), Ala572 → Val (14, 16), Thr577 → Ile (13, 16), and Asp578 → Gly (13, 14) were examined for IP, induction. These mutant receptors were capable of CG-dependent IP, induction without affecting the affinity. The maximum IP induction levels were, however, 50-75% of that of the wild-type receptor. In these studies, IP1, IP2, and IP3 were not analyzed. It is not surprising that their substitutions show partial impacts on cAMP induction and a marginal effect on IP induction since all of the amino acids reside in the cytoplasmic half of TM 6. This region is adjacent to the C-terminal half of cyto loop 3, which is thought to couple with Go protein (22), the linkage to adenylyl cyclase. It is unknown, at the present time, where Go couples to the LH/CG receptor. However, cyto loop 1 of the thyroid-stimulating hormone receptor with cAMP and IP signaling has been implicated as a Go coupling site (9, 25).

The serum levels of LH in humans are in the range of several hundred-fold lower than the EC50 of IP induction by the wild-type receptor (Fig. 3). However, intracellular Ca2+ induction (which is stimulated by CG) shows significantly higher sensitivity in the range of ~100 pm in transfected L cells (26). This result suggests the physiological relevance of the IP/Ca2+ system.

In conclusion, our data demonstrate that the generation of the cAMP signal and the IP signal independently involves Lys583. The surface location of Lys583 implies that both signals originate from the cell surface, presumably near or at the hormone binding site. Furthermore, the two signals diverge and transduce through Lys583 at the cell surface, the cAMP signal presumably toward a Go, coupling site and the IP signal to a Go, site in cyto loops (9, 22, 23).

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Differential Signal Generation of LH/CG Receptor

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