Mediation of Cyclic AMP Signaling by the First Intracellular Loop of the Gonadotropin-releasing Hormone Receptor*

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The gonadotropin-releasing hormone (GnRH) receptor, which is a unique G protein-coupled receptor without a C-terminal cytoplasmic domain, activates both inositol phosphate (InsP) and cAMP signaling responses. The function of the highly basic first intracellular (1i) loop of the GnRH receptor in signal transduction was evaluated by mutating selected residues located in its N and C termini. Replacements of Leu58, Lys59, Gln61, and Lys62 at the N terminus, and Leu73, Ser74, and Leu80 at the C terminus, caused no change in binding affinity. The agonist-induced InsP and cAMP responses of the Q61E and K59Q,K62Q receptors were also unaffected, but the L58A receptor showed a normal InsP response and an 80% decrease in cAMP production. At the C terminus, the InsP response of the L73R receptor was normal, but cAMP production was reduced by 80%. The EC50 for GnRH-induced InsP responses of the S74E and L80A receptors was increased by about one order of magnitude, and the cAMP responses were essentially abolished. These findings indicate that cAMP signaling from the GnRH receptor is dependent on specific residues in the 1i loop that are not essential for activation of the phosphoinositide signaling pathway.

A wide variety of neurotransmitters, peptide and protein hormones, chemokines, growth factors, and other ligands elicit specific cellular responses by binding to plasma membrane receptors that are coupled to one or more heterotrimeric guanine nucleotide binding/regulatory proteins (G proteins). The primary signaling pathways for many of these receptors have been elucidated (1, 2). Agonist binding to a specific receptor on the cell surface causes a conformational change in the receptor that allows it to interact with its cognate G protein, stimulating guanine nucleotide exchange on the G protein subunit of the G protein. The release of the α subunit-GTP and βγ subunits from the receptor-G protein complex, and the activation of effector systems including phospholipase C, adenylyl cyclase, and ion channels, regulate the intracellular levels of inositol phosphate, calcium, cyclic AMP, and other second messengers.

Although most G protein-coupled receptors (GPCRs) share a common structure, based on seven membrane-spanning domains, relatively little is known about the functional significance of this arrangement. It probably provides structural and functional integrity to the receptor, and the presence of several conserved amino acid residues in the transmembrane regions and cytoplasmic loops may reflect their role in agonist-induced G protein coupling and signal generation. Mutagenesis and chimeric studies with several receptor-G protein pairs, including the β-adrenergic-Gαs, muscarinic acetylcholine-Gαq, angiotensin II-Gαq, and rhodopsin-Gαs, have implicated the N- and C-terminal portions of the third intracellular (3i) loop of the receptors in G protein activation and signal transduction (3–11). In the case of the thyroid stimulating hormone (TSH) receptor-Gαs, dopamine (D1) receptor-Gαs, and rhodopsin-Gαs, regions within the cytoplasmic tail have also been found to interact with G proteins (12–14).

An analysis of the structural determinants in the IGF-II receptor, and GPCRs that are associated with Gαi and GαJ activation (15) revealed the following criteria: (a) at least two basic residues at the N terminus; (b) either a BBXXB or BBXB motif (where B is a basic amino acid and X is any amino acid) at the C terminus; and (c) a sequence length of 10–26 amino acids. The structural motifs satisfying G protein activation requirements for several GPCRs were conserved in the 2i and 3i loops, and in the C-terminal tail (15). Using synthetic peptides with these structural characteristics, multiple Gαi-activating regions were identified in M4 muscarinic cholinergic and α1-adrenergic receptors (15). Recently, a synthetic peptide corresponding to the cytoplasmic tail of the rat testicular follicle-stimulating hormone receptor that satisfied these criteria was shown to modulate G protein signaling (16). However, these studies did not identify functionally important specific residues within the structural motifs.

The gonadotropin-releasing hormone (GnRH) receptor is a member of the GPCR superfamily and has several unique structural features. These include (a) the absence of a cytoplasmic C-terminal tail; (b) the replacement of Tyr by Ser in the conserved GPCR “signature” motif DRY located at the junction of transmembrane domain (TMD) III and the 2i loop; (c) the interchange of conserved Asp and Asn residues in TMDs II and VII; and (d) the presence of a relatively long and highly basic 1i loop. These characteristics are conserved in the GnRH receptors of all mammalian species sequenced to date (17, 18). The primary structural of the 1i loop of the GnRH receptor, which is coupled to both phosphoinositide hydrolysis and cAMP generation, meets the criteria for G protein activation by other receptors that are coupled to Gαs and Gαi (15, 16). The 18 amino acid 1i loop of the GnRH receptor contains two basic amino acid residues at the N terminus (Lys59 and Lys62) and a BBXXB motif at the C terminus (Lys71-Lys-Ser-Glu-Lys75) (see Fig. 1).

The present study was performed to determine whether these regions of the 1i loop are involved in GnRH-induced signal transduction and receptor internalization. The roles of the N

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‡ The abbreviations used are: GPCR, G protein-coupled receptor; GnRH, gonadotropin-releasing hormone; cAMP, cyclic AMP; Ins (1,4,5)P3, inositol 1,4,5-trisphosphate; InsP2, inositol bisphosphate; TSH, thyroid stimulating hormone; TMD, transmembrane domain; WT, wild-type; CCK, cholecystokinin.
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EXPERIMENTAL PROCEDURES

Materials—GnRH and its agonist (des-Gly10-[d-Ala6]GnRH N-ethylamidine, GnRH-Ag) and antagonist ([p-Glu1,-Phe2,-Trp3,6]GnRH) analogs were obtained from Peninsula Laboratories, Inc. (Belmont, CA). LipofectAMINE and Opti-MEM media were purchased from Life Technologies, Inc., cell culture related products were from Biofluids (Rockville, MD), restriction and DNA-modifying enzymes were from New England BioLabs (Beverly, MA), and Sequenase II from United States Biochemical Corp. Oligonucleotide primers for site-directed mutagenesis were obtained from Peninsula Laboratories, Inc. (Belmont, CA). Materials for DNA purification, chromaffin or analytical grade quality. AG-1-X8 resin (100–200 mesh formate form) and Poly-Prep chromatography columns for anion exchange chromatography were obtained from Bio-Rad. All other reagents were of high performance liquid chromatography or analytical grade quality. myo-[1-3H]Inositol (80–100 Ci/mmol) was from Amersham Pharmacia Biotech. [125I]des-Gly10-[d-Ala6]GnRH N-ethylamide (125I-GnRH-Ag) and [3H]S-cAMP-TME were prepared by Covance Laboratories Inc. (Vienna, VA).

Construction of Wild-type and Mutant GnRH Receptors—The 1220-base pair GnRH receptor cDNA subcloned into pcDNAI/Amp at the XhoI site (19) was used as a template for creating site-directed mutations according to the method of Kunkel et al. (20) using a Muta-Gene phagemid in vitro mutagenesis kit. Mutations were identified by the dideoxy sequencing method of Sanger et al. (21) using Sequenase II.

Receptor Expression in COS-7 Cells—Wild-type and mutant GnRH receptors were transiently expressed in COS-7 cells. To measure inositol phosphate and cAMP responses, or [125I]GnRH-Ag binding to intact cells, cultures were seeded in 24-well plates (Costar Corp.) at a density of 4 × 104 cells/well. The plated cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum containing 100 units/ml penicillin and 100 μg/ml streptomycin (Pen-Strep) at 37 °C in an atmosphere consisting of 5% CO₂, 95% humidified air. At 70–80% confluence, the cells were transfected in 0.5 ml of serum-free Opti-MEM I medium with 1 μg of wild-type or mutant plasmid DNA and 6–8 μg of LipofectAMINE/well. Six hours later, the medium was replaced with fresh medium and cultures were maintained for 48 h before use in ligand binding and functional assays.

Receptor Binding and Internalization Assays—The binding affinity and abundance of the mutant receptors were determined in transfected COS-7 cells incubated with 2 nM [125I]GnRH-Ag in binding medium (M199 containing 25 mM HEPES and 0.1% bovine serum albumin) in the absence or presence of increasing concentrations of unlabelled peptide for 4 h at 4 °C. The cells were then rapidly washed twice with ice-cold phosphate-buffered saline (pH 7.4) and solubilized in 0.2 M NaOH, 1% SDS solution. The cell-associated radioactivity was measured by γ-spectrometry. All time studies were performed in duplicate on at least three occasions, and displacement curves were analyzed for binding affinity and capacity by the LIGAND program using a one-site model (22). Surface expression of most of the mutant receptors ranged from 50 to 82% of that of the wild-type (WT) receptor, except that L73R receptors were better expressed (150% of WT) and S74E receptors were poorly expressed (30% of WT). The expression levels for the L58A, Q61E, K59Q,K62Q, and L80A receptors were 82, 51, 44, and 71%, respectively, of the wild-type. The 100% level of wild-type receptor expression corresponded to 710 fmol/mg protein. Irrespective of differences in plasma membrane binding sites, the mutant receptors had similar binding affinities with apparent Kd values in the range of 1.6 to 3.1 nM for the various mutants. The Kd of the wild-type receptor was 1.8 ± 0.2 nM (n = 3). The nonspecific binding for the [125I]GnRH-Ag determined in the presence of unlabeled agonist (1 μM) for wild-type or mutant receptors was always less than 5% of the respective total binding. For internalization assays, transfected COS-7 cells were washed once with binding medium before the addition of 2 nM [125I]labeled GnRH agonist. Nonspecific binding was determined in the presence of a 1000-fold excess of the unlabeled GnRH agonist. After incubation at...
37 °C for the indicated times, the cells were washed twice with ice-cold phosphate-buffered saline (pH 7.4) and incubated with 1 ml of 50 mM acetic acid, 150 mM NaCl (pH 2.8) for 12 min to remove surface-bound tracer. The acid-released radioactivity was collected to determine the receptor-bound radioactivity, and the internalized (acid-resistant) radioligand was quantitated after solubilizing the cells in NaOH/SDS solution. Radioactivities were measured by γ-spectrometry, and the internalized radioligand at each time point was expressed as a percent of the total (acid-resistant + acid-released) binding.

Inositol Phosphate Production—COS-7 cells were labeled 24 h after transfection by incubation in inositol-free Dulbecco’s modified Eagle’s medium containing 20 μCi/ml [3H]inositol as described previously (19). After 24 h of labeling, cells were washed with inositol-free M199 medium and preincubated in the same medium containing 10 mM LiCl for 30 min at 37 °C and then stimulated with increasing doses of GnRH (10^{-10} to 10^{-6} M) for 20 min. Incubations were terminated by the addition of ice-cold perchloric acid (5% v/v final concentration). The inositol phosphates were extracted and separated by anion exchange chromatography as described previously (23), and their radioactivities (InsP_2 + InsP_3) were measured by liquid scintillation β-spectrometry.

cAMP Production—After 48 h of transfection, cells expressing WT or mutant receptors were incubated in the presence of increasing concentrations of GnRH (10^{-10} to 10^{-6} M) in serum-free medium (1:1 Dulbecco’s modified Eagle’s medium/F-12) containing 0.1% BSA, 30 mg/liter bacitracin, and 1 mM isobutylmethylxanthine for 30 min at 37 °C. Incubations were terminated by aspirating the medium and freezing the cells to −20 °C. The samples were processed for extraction, and intracellular cAMP was measured by radioimmunoassay (24) using a specific cAMP antisera at a final dilution of 1:5,000. This assay showed no cross-reaction with cGMP, 2’,3’-cAMP, ADP, GDP, CTP, or isobutylmethylxanthine. The data were analyzed by the GraphPad PRISM program using nonlinear regression analysis and the best fitted sigmoidal dose response.

**RESULTS**

Expression of 1i Loop Mutant GnRH Receptors in COS-7 Cells—Six substitution mutations were created in the 1i loop of the mouse GnRH receptor, as shown in Fig. 1. Two modifications at the N terminus of the loop included mutation of residues Gln^{61} to Glu, and Lys^{68} to Glu (a double mutant). At the C terminus, residues Leu^{73} and Ser^{74} in the BBXXB motif were mutated to Arg and Glu, respectively. In addition, leucine residues (Leu^{58} and Leu^{60}) located at the juxtamembrane portions of the N and C termini of the 1i loop, respectively, were individually replaced with alanine. Measurement of 125I-GnRH-Ag binding in intact COS-7 cells transfected with WT or mutant GnRH receptors, to determine the cell-surface expression and the functional integrity of the expressed receptors, indicated that the WT and modified receptors bound the radioligand with high affinity. Scatchard analysis of the binding data yielded linear plots, reflecting a single class of GnRH-binding sites with similar dissociation constants (see “Experimental Procedures”).

Effect of 1i Loop Mutations on GnRH-mediated Inositol Phosphate Signaling—The ability of the mutant receptors to couple to phospholipase C via γ_{i}γ_{j} proteins was determined by measuring the inositol phosphate (InsP) responses of transfected COS-7 cells stimulated with 10^{-10} to 10^{-6} M GnRH in the presence of 10 mM LiCl. Under these experimental conditions, the major accumulated products of phosphoinositide hydrolysis in GnRH receptor-transfected cells are InsP_2 and InsP_3 (19). The GnRH-induced InsP responses mediated by receptors with mutations at the N terminus (L58A, Q61E, and K59Q,K62Q) were similar to those of the WT receptors, and their EC_{50} values were 1.5–3.6 nM; the EC_{50} for the WT receptor was 1.4 ± 0.2 nM (n = 4) (Fig. 2A). Among the C-terminal mutant receptors the InsP response of the L73R receptor was normal, with EC_{50} of 1.0 nM. For the S74E and L80A receptors, agonist potency was moderately impaired, as indicated by increases of EC_{50} to 9 and 15 nM, respectively (Fig. 2B). The magnitudes of the InsP responses elicited by a saturating concentration of GnRH (1 μM) for the mutant receptors were 80–110% of that for the wild-type response.

Because the plasma membrane binding sites of cells expressing the mutant GnRH receptors showed significant differences (see “Experimental Procedures”), corrections for variations in expression levels of the mutant receptors were made by normalizing their signaling responses to the number of cell-surface binding sites measured in the same experiment. We have previously shown a linear relationship between the measured receptor sites and the InsP responses to GnRH stimulation in receptor-transfected cells (23). Normalization of the data based on receptor number showed that the InsP responses mediated by the L73R and L80A receptors were similar to that of the WT.
receptor. The InsP responses of the L58A, Q61E, K59Q,K62Q, and S74E mutants were augmented relative to the WT receptor, suggesting that the “activated” state of these receptors couples more efficiently to Gq than the activated wild-type receptor. These results indicated that modifications at the N and C termini of the first loop of the GnRH receptor do not greatly affect the ability of the receptors to activate phospholipase C.

**Effect of 1i Loop Mutations on GnRH-mediated cAMP Production**—The ability of the wild-type GnRH receptor to activate adenylyl cyclase is shown in Fig. 3. Treatment with increasing concentrations of GnRH (10^{-9}–10^{-6} M) elicited a continuous rise in cAMP accumulation with a single slope (Hill coefficient 0.9). The maximal response was at least 2-fold over basal levels, with an estimated EC_{50} of 9 nM. Cells transfected with vector alone showed no increase in GnRH-induced cAMP production (Fig. 3). The receptor specificity of the cAMP response was demonstrated by the ability of a GnRH antagonist (1 μM), applied prior to stimulation with GnRH, to abolish cAMP production (Fig. 4); the antagonist itself elicited no cAMP response. These results indicated that the GnRH receptors expressed in COS-7 cells are functionally coupled not only to the phosphoinositide/phospholipase C pathway, but also activation of the adenylyl cyclase signaling pathway. The ability of the 1i loop mutant receptors to mediate GnRH-induced activation of adenylyl cyclase, as judged by cAMP production, was also examined in transfected COS-7 cells. The dose-dependent responses are shown in Figs. 5A and 6A. Among the N-terminal mutants (Fig. 5), the cAMP response of cells expressing the L58A receptor was markedly impaired, with reduction of 80% from that of the wild-type receptors in response to a maximal (1 μM) concentration of GnRH (Fig. 5B). In contrast, the cAMP response for the Q61E receptor did not differ from that of the WT receptor, and K59Q,K62Q receptors were about 20% less effective than the WT receptor. The effects of the C-terminal mutants on the cAMP response were much more pronounced, as shown in Fig. 6. cAMP production for the L73R and S74E receptors was reduced by 80–90% of the WT response, and the L80A receptors failed to activate adenylyl cyclase in GnRH-treated cells (Fig. 6B). These results indicated that modifications of the first loop, specifically at its C terminus, dramatically impair the ability of the GnRH receptors to activate adenylyl cyclase activity.

**Effect of 1i Loop Mutations on GnRH Receptor Internalization**—The effects of mutations on ligand-induced internalization were evaluated by measuring the kinetics of 125I-GnRH-Ag uptake over a period of 60 min at 37 °C in cells expressing wild-type or mutant receptors. A direct comparison between the wild-type and mutant receptors was made by plotting the percent of bound radioligand that was internalized with increasing time of incubation (Fig. 7). Most of the mutant receptors at the N terminus, and L73R at the C terminus, were internalized less efficiently than the WT receptor. The results indicate that modifications of the first loop, specifically at its C terminus, dramatically impair the ability of the GnRH receptors to activate adenylyl cyclase activity.

**Role of First Intracellular Loop in GnRH Receptor Function**

FIG. 3. Agonist-induced cAMP production mediated by the wild-type GnRH receptor. COS-7 cells expressing GnRH receptors were incubated for 30 min in serum-free medium and then stimulated with increasing concentrations of GnRH for 30 min at 37 °C. Cyclic AMP was extracted and assayed as described under “Experimental Procedures.” The data shown are representative of three similar experiments.

FIG. 4. Blockade of agonist-induced cAMP response by a GnRH antagonist. Prior to stimulation with GnRH, cells were treated with a GnRH antagonist (1 μM, 30 min). The data shown are representative of three similar experiments.

FIG. 5. Effects of site-directed mutations in the N terminus of the 1i loop of the GnRH receptor on GnRH-induced cAMP responses. COS-7 cells expressing WT and mutant GnRH receptors were stimulated with GnRH (10^{-9}–10^{-6} M). Panel A, cAMP dose-response curves of WT and mutant receptors. These data are representative of three similar experiments each performed in quadruplicate. Panel B, the cAMP responses normalized to the number of 125I-GnRH-Ag binding sites. These data were calculated after subtracting the respective basal levels in nonstimulated cells from the maximal response obtained after stimulation with 1 μM GnRH and then normalized to the number of expressed receptor sites. Results are expressed as percent of the wild-type response. For values shown, the S.E. values were less than 10% of the mean.
nus, showed internalization kinetics similar to that of the wild-type. However, internalization of the S74E and L80A mutants was reduced by about 50% after 1 h (Fig. 7B).

DISCUSSION

To gain insight into the structure/function relationships of the GnRH receptor, we constructed a series of GnRH receptors with mutations in the first intracellular loop and compared their binding characteristics and second messenger signaling properties with those of wild-type receptors transiently expressed in COS-7 cells. These analyses revealed that mutations of residues Leu58, Lys59, Gln61, and Lys62 at the N terminus, and Leu73, Ser74, and Leu80 at the C terminus, of the 1i loop had no significant effect on the Gq-coupled phospholipase-catalyzed inositol phosphate production. However, Gs-coupled adenylyl cyclase-activated cAMP production was severely impaired by substitutions at the carboxyl-terminal region of the loop. These findings demonstrate that residues in the 1i loop are required for selectively coupling to adenylyl cyclase but are not essential for coupling to the phospholipase C pathway. The present studies on the GnRH receptor describe the first mutational analysis of this structural motif previously identified in other GPCRs by the synthetic peptide strategy (15, 16).

A search of the primary sequence of the GnRH receptor for putative G protein-coupling regions, based on conserved sequence motifs with confirmed G protein coupling functions (for Gs and Gi) within the sequences of other receptors, indicated that the 1i loop may act as a receptor-G protein activation region. Application of the structural criteria used to identify G protein activating domains in a number of Gi/Gs coupled receptors (15) to the GnRH receptor revealed that the 1i loop has at least two basic residues at the N terminus and a BBXXB sequence motif at the C terminus. In other receptors, this motif is usually present in the 2i or 3i loop, or in the C-terminal cytoplasmic tail (15, 16). However, in the tail-less GnRH receptor, it is located in the 1i loop, which is longer and richer in basic residues than most other receptor loops in this position. The present studies on the GnRH receptor describe the first mutational analysis of this structural motif previously identified in other GPCRs by the synthetic peptide strategy (15, 16).

Although it is well established that most of the biological actions of GnRH are mediated by Gq-coupled pathways, leading to elevations of InsP3, diacylglycerol, and intracellular Ca2+ (17), increased cAMP signaling may also mediate physiological responses in gonadotropes. Previous reports have suggested a
physiological role of cAMP as a mediator of GnRH actions in the pituitary gland (25). Also, in hypothalamic neuronal GT1–7 cells, the elevation of intracellular cAMP levels by forskolin or application of 8-bromo-cAMP was found to mimic GnRH receptor activation by stimulating neurosecretion (24). Some recent reports have shown that GnRH can activate the Gs-coupled adenylyl cyclase-activated unbalanced by adding an acidic or basic residue at the C terminus of the receptor (located at the same relative position as Ala 623 in the rat parathyroid hormone receptor). For example, GnRH-stimulated cAMP production in GH2 cells stably expressing the rat GnRH receptor was found to mediate prolactin release through a cholera toxin-sensitive G protein (26). In addition, activation of GnRH receptors in GT1 cells not only stimulated phospholipase C activity but also increased cAMP production in a biphasic manner, suggesting that the GnRH receptor can interact with both adenylyl cyclase stimulatory and inhibitory G proteins. The present results suggest that the structural determinants that are associated with Gs/Gi activation are not essential for Gs-coupled inositol phosphate formation, but are important for Gi-coupled cAMP production. In this regard, our results are in accordance with observations on Gs- and Gi-coupled receptors studied by synthetic peptide strategies (15, 16). In addition, the present study demonstrates that these structural criteria are not involved in the activation of phosphoinositide/calcium signaling pathway, and identifies specific amino acid residues involved in Gi coupling. Clearly, positive charges are important for the receptor-Gi protein interaction. If the overall charge is unbalanced by adding an acidic or basic residue at the C terminus of the 1i loop, the Gi-coupled adenylyl cyclase-activated cAMP response is severely impaired. Thus, it is probable that these structural regions/residues confer specificity to the receptor-Gi protein interaction (Gs versus Gi).

Upon agonist binding, GnRH receptor activation causes a Gi-mediated increase in protein kinase C activity and elevation of intracellular Ca2+ levels. It is possible that the activation of adenylyl cyclase observed in GnRH receptor-expressing COS-7 cells (Figs. 3 and 4) is caused by Gia released during Gi activation, increased protein kinase C activity, or elevated Ca2+ levels (27). However, the largely unchanged profile of InsP levels (27). However, these residues do not appear to be required for the receptor-Gi protein interaction. If the overall charge is unbalanced by adding an acidic or basic residue at the C terminus of the 1i loop, or a combination of positive charges, is important for coupling the GnRH receptor to Gi and adenylyl cyclase. However, these residues do not appear to be required for ligand-induced internalization of the receptor.

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