Identification of Ser\textsuperscript{153} in ICL2 of the Gonadotropin-releasing Hormone (GnRH) Receptor as a Phosphorylation-independent Site for Inhibition of G\textsubscript{q} Coupling*

Sharon Shacham\textsuperscript{‡‡}, Maya N. Cheifetz\textsuperscript{‡‡}, Mati Fridkin\textsuperscript{‡}, Adam J. Pawson\textsuperscript{‡}, Robert P. Millar\textsuperscript{‡}, and Zvi Naor\textsuperscript{‡‡‡}

From the \textsuperscript{‡}Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel, the \textsuperscript{‡‡}Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel, and the \textsuperscript{‡‡‡}Human Reproduction Sciences Unit, Medical Research Council, the University of Edinburgh Chancellor’s Building, 49 Little France Crescent, Edinburgh EH16 4SB, Scotland, United Kingdom

Type I gonadotropin-releasing hormone (GnRH) receptor (GnRHR) is unique among mammalian G-protein-coupled receptors (GPCRs) in lacking a C-terminal tail, which is involved in desensitization in GPCRs. Therefore, we searched for inhibitory sites in the intracellular loops (ICLs) of the GnRHR. Synthetic peptides corresponding to the three ICLs were inserted into permeabilized αT3-1 gonadotrope cells, and GnRH-induced inositol phosphate (InsP\textsubscript{3}) formation was determined. GnRH-induced InsP\textsubscript{3} production was potentiated by ICL2 > ICL3 but not by the ICL1 peptides, suggesting they are acting as decoy peptides. We examined the effects of six peptides in which only one of the Ser or Thr residues was substituted with Ala or Glu. Only substitution of Ser\textsuperscript{153} with Ala or Glu ablated the potentiating effect upon GnRH-induced InsP\textsubscript{3} elevation. ERK activation was enhanced, and the rate of GnRH-induced InsP\textsubscript{3} formation was about 6.5-fold higher in the first 10 min in COS-1 cells that were transfected with mutants of the GnRHR in which the ICL2 Ser/Thr residues (Ser\textsuperscript{151}, Ser\textsuperscript{153}, and Thr\textsuperscript{145}) or only Ser\textsuperscript{153} was mutated to Ala as compared with the wild type GnRHR. The data indicate that ICL2 harbors an inhibitory domain, such that exogenous ICL2 peptide serves as a decoy for the inhibitory site (Ser\textsuperscript{153}) of the GnRHR, thus enabling further activation. GnRH does not induce receptor phosphorylation in αT3-1 cells. Because the phosphomimetic ICL2-S153E peptide did not mimic the stimulatory effect of the ICL2 peptide, the inhibitory effect of Ser\textsuperscript{153} operates through a phosphorylation-independent mechanism.

The desensitization mechanism of GPCRs\textsuperscript{1} involves binding of the agonist to the receptor, which not only causes activation of the effector but also facilitates phosphorylation of the receptor by second messengers-activated kinases or by G-protein-coupled receptor kinases (GRKs) on specific sites within the C-terminal tail. The phosphorylation enables binding of β-arrestin, which prevents further effector activation and targets the desensitized receptor for internalization via clathrin-coated vesicles that are pinched off from the plasma membrane by dynamin (1, 2). The receptor is then dephosphorylated by a GPCR phosphatase (3) and can be targeted to lysosomes for degradation or recycled to the cell surface (1, 2).

A striking feature of mammalian type I GnRHR is the absence of a C-terminal tail (4). Therefore, it is thought that the GnRHR does not undergo C-terminal tail phosphorylation, rapid desensitization, and internalization (for review see Ref. 5). Therefore, the known desensitization of gonadotropin secretion observed during sustained GnRHR administration is attributed to down-regulation of inositol 1,4,5-trisphosphate receptors, desensitization of Ca\textsuperscript{2+} mobilization, reduction in the number of GnRHR, and G\textsubscript{q11} expression and attenuation of phospholipase D activation and arachidonic acid release (5). In addition, prolonged GnRH treatment down-regulates PKC, Ca\textsuperscript{2+}, and Ca\textsuperscript{2+}-dependent signaling (6). Because the GnRHR lacks a C-terminal tail, we reasoned that other structural components, such as the ICLs, might be involved in signal termination of the GnRHR as outlined above.

Synthetic peptides are important tools for understanding the sites and mechanisms of receptor/G-protein interactions. It was shown that peptides derived from the ICL2, ICL3, and ICL4 loops of rhodopsin disrupt the ability of G\textsubscript{i} to stabilize the active signaling conformation of rhodopsin and metarhodopsin II (7, 8). In another study it was shown that a dimer of peptides from the C-terminal and N-terminal regions of ICL3 of the α\textsubscript{2A}-adrenergic receptor affect the stimulation of the guanine-nucleotide exchange protein of G\textsubscript{i} (9). Although a motif scan of the human GnRHR identifies several potential phosphorylation sites in the intracellular loops, including sites for protein kinase A (Ser\textsuperscript{74}, Thr\textsuperscript{84}, and Thr\textsuperscript{285}), PKC (Thr\textsuperscript{40}, Thr\textsuperscript{42}, Thr\textsuperscript{51}, Ser\textsuperscript{74}, Thr\textsuperscript{84}, Ser\textsuperscript{118}, Thr\textsuperscript{265}, and Thr\textsuperscript{274}), and calmodulin-dependent kinase 2 (Thr\textsuperscript{285}), it is not known whether these sites are functional and involved in receptor regulation (4).

We have therefore utilized synthetic peptides corresponding to the ICLs of the mammalian type I GnRHR in order to identify potential inhibitory sites involved in GnRHR function. Here we report that Ser\textsuperscript{153} is a key residue in an inhibitory domain in ICL2, which exerts its inhibitory effect in a phosphorylation-independent manner.
The mutations were performed using separate primers. The plasmid with the influenza HA epitope inserted at the KpnI and XhoI sites of the cDNA of the influenza hemagglutinin (HA) epitope-tagged rat GnRHR in pcDNAs plasmid was kindly provided by Dr. Y. Koch (The Weizmann Institute, Rehovot, Israel). Mouse monoclonal anti-HA epitope tag, secondary antibody goat anti-mouse, mouse monoclonal anti-active (doubly phosphorylated) ERK, and polyclonal antibodies to general ERK were from Sigma. Secondary antibody goat anti-rabbit was purchased from Jackson ImmunoResearch. Antibodies to general ERK were from Cell Signaling, anti-active (doubly phosphorylated) ERK, and polyclonal antibodies to general ERK were from Sigma. Secondary antibody goat anti-rabbit was purchased from Jackson ImmunoResearch.

**Immunoblots**—Membranes were prepared from COS-1 cells that were removed from dishes by scraping followed by homogenization in a Dounce homogenizer (30 strokes). The nuclei were then pelleted by centrifugation at 750 × g for 15 min. The supernatant was collected and centrifuged again at 60,000 × g for 30 min to obtain the pellet. The pellet was collected and resuspended in sample buffer for protein separation on 10% SDS-PAGE, followed by Western blotting with mouse monoclonal antibody directed at the HA epitope tag (10). The blots were developed with alkaline phosphatase or horseradish peroxidase-conjugated anti-mouse or anti-rabbit Fab antibodies (Jackson ImmunoResearch).

**Receptor Internalization**—Forty-eight hours after transfection, the cells were washed in ice-cold buffer I and then incubated with 100,000 cpm 125I-GnRH-A for 3 h on ice. The cells were then moved to a 37 °C water bath and incubated for the indicated times to allow internalization, without removing the radiolabeled peptide from the medium. After the incubation, the cells were transferred to an ice bath and washed twice with PBS. Externalized ligand was analyzed by a VMA-assay: 32P-montmorillonite (1 M myo-inositol, 10 mM phenylacetic acid, 150 mM/mM NaCl), whereas the internalized ligand was measured by solubilizing the cells with 0.1 mol/liter NaOH as above.

**Activation of Mitogen-activated Protein Kinase Cascades**—Cells were grown in 6-well plates, serum-starved (0.5% FCS) for 16 h, and later stimulated with GnRH, and the cells were washed twice with ice-cold PBS and once with ice-cold buffer A (50 mM β-glycerophosphate, 1.5 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate). Cells were harvested in 0.3 ml of buffer H (buffer A containing 1 mM benzenamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride) followed by sonication (two times for 7 s, 40 watts) and centrifugation (15,000 × g, 15 min, 4 °C). The supernatants containing the collected aliquots (30 μg/sample) were separated on 10% SDS-PAGE, followed by Western blotting with mouse monoclonal anti-phospho-ERK. Total ERK was detected with a polyclonal antibody as a control for sample loading. The blots were developed with alkaline phosphatase or horseradish peroxidase-conjugated anti-mouse or anti-rabbit Fab antibodies (Jackson ImmunoResearch). The blots were autoradiographed on Kodak X-100 films, and the phosphorylation was quantified by densitometry (Bio-Rad 690 densitometer).

**RESULTS**

GnRH-induced InS End Formation Is Attenuated by Prior Exposure of the Cells to a GnRH Challenge—Pretreatment of αT3-1 cells with GnRH for 1 or 2 h abolished the ability of a second stimulus of GnRH to elevate InS levels (Fig. 1A). To gain
Further insight into the kinetics of the effect, a kinetic response of GnRH-induced InsP elevation was performed. αT3-1 cells were treated with GnRH for varying times, and InsP levels were determined. As shown in Fig. 1B, the rate of elevation of InsP levels in the first 10 min was 10-fold higher than in the following 10–60 min of incubation (1.1- and 0.1-fold/min, respectively). The results suggest that an inhibitory process at the receptor level, or at a more distal downstream signaling events, was initiated after 10 min of ligand stimulation. Based on these results, we used GnRH elevation of InsP after 30 min of treatment as a marker for receptor activation in the rest of the experiments.

**Synthetic Peptides Corresponding to ICL2 and ICL3 but Not ICL1 of the GnRHR Potentiate InsP Formation by GnRH**—As mentioned before, one of the unique structural features of the mammalian type I GnRHR is the absence of a C-terminal tail. Therefore, to test the hypothesis that the ICLs may be involved in GnRHR regulation, synthetic peptides corresponding to the ICLs of the GnRHR were synthesized. Because ICL3 of the GnRHR is relatively long, we prepared separate peptides and with other ligands known to interact with pituitary gonadotropins, namely pituitary adenylate cyclase-activating polypeptide (PACAP), oxytocin, and endothelin (each at 100 nM). Only GnRH-induced InsP production was potentiated by the ICL2 peptide (not shown), indicating specific interaction of the inhibitory site with the GnRHR.

We then tested whether the synthetic peptides that correspond to ICL1, ICL2, or ICL3 affect GnRHR activation. Permeabilized αT3-1 cells were incubated with the peptides (each at 100 μM). After 1 h of recovery, the cells were treated with or without GnRH for 30 min, and InsP levels were determined. None of the synthetic peptides altered basal InsP levels. The ICL2 peptide gave a marked enhancement of the GnRH response (p < 0.001; Fig. 2). The two peptides comprising the N and the C termini of ICL3 induced a smaller enhancement of GnRH-induced InsP elevation. In contrast, ICL1 had a small inhibitory effect. The data suggest that ICL-2 and to a lesser degree ICL3, but not ICL1, in the GnRHR harbor inhibitory sites. Therefore, the exogenously added ICL2 and ICL3 peptides may serve as "decoy peptides" and protect the receptor ICL inhibitory sites from being targeted by intracellular proteins involved in desensitization.

**Preincubation of αT3-1 Cells with Increasing Concentrations of the ICL Peptides**—The ICL peptides were then performed (Fig. 3). The peptides had no effect on basal InsP levels. The ICL1 peptide had no effect on GnRH-induced InsP elevation (Fig. 4A). However, the ICL2 peptide enhanced GnRH-stimulated InsP production in a dose-response manner (Fig. 3B). The two peptides ICL3N and ICL3C displayed a concentration-dependent potentiating effect on GnRH-induced InsP elevation (Fig. 3, C and D), albeit less pronounced as that obtained with the ICL2 peptide. Because the maximal effect was observed at a concentration of 100 μM of the peptides, this concentration was used for the rest of the experiments. This is a relatively high concentration compared with GnRHR in cells, and probably reflects the high flexibility of small synthetic peptides and their limited ability to mimic the constrained loop structure in the receptor. We reasoned that if the peptides are titrating an inhibitor such as an RGS protein, then other Q-β-linked receptors are also likely to be affected. We therefore repeated the experiment with GnRH (as a positive control) and with other ligands known to interact with pituitary gonadotropins, namely pituitary adenylate cyclase-activating polypeptide (PACAP), oxytocin, and endothelin (each at 100 nM). Only GnRH-induced InsP production was potentiated by the ICL2 peptide (not shown), indicating specific interaction of the inhibitory sites.
Ser<sup>153</sup> Plays a Key Role in the Protective Effect of ICL2—The putative importance of Ser and Thr residues in ICL2 of other GPCRs (15, 16) led us to synthesize modified peptides, in which the Ser or Thr residues were changed to Ala, and to use these peptides to test whether one of the Ser/Thr residues is responsible for the stimulatory effect of the ICL2 peptide. Because the Ser and Thr residues are potential targets for various kinases, which may participate in desensitization, we also synthesized modified phosphomimetic peptides, in which the Ser or Thr residues were changed to Glu, which is predicted to mimic phosphorylation. The sequences and abbreviations of the peptides are given in Table I. T3-1 cells were permeabilized and incubated with ICL2-WT, ICL2-Ala, or ICL2-Glu, in which one Thr and two Ser residues were replaced by Ala or Glu, respectively (Table I). Results in Fig. 5 show that as before the addition of the ICL2-WT peptide enhanced GnRH-induced InsP formation. However, the protective effect of the ICL2-WT peptide was abolished when the cells were incubated with the ICL2-Ala or the ICL2-Glu peptides. The results suggest that one or more of the Ser or Thr residues in ICL2 of the GnRHR are involved in the potentiation effect of ICL2 on the GnRHR response, and this is phosphorylation-independent. Therefore, six peptides in which only one of the Ser or Thr residues was replaced with Ala or Glu were inserted into permeabilized aT3-1 cells, and GnRH-induced InsP elevation was determined (Fig. 6). As before, addition of ICL2-WT enhanced the GnRH response, and this potentiating effect was lost upon the insertion of ICL2-Ala or ICL2-Glu. Of the peptides with single substitutions, only ICL2-T144A and ICL2-T144E retained the potentiating effect of the ICL2-WT. The peptides ICL2-S151A and ICL2-S151E enhanced the GnRH response, albeit to a lesser degree than the ICL2-WT. Only replacement of Ser<sup>153</sup> to Ala or Glu (ICL2-S153A and ICL2-S153E) restored the inhibitory effect of the ICL2-Ala and ICL2-Glu peptides. The observation that the Ala and the Glu mutants of ICL2 gave similar results suggests that the Ser or the Thr residues of ICL2 are not phosphorylated during receptor activation and are responsible for desensitizing the receptor by a phosphorylation-independent mechanism. Indeed, Ser<sup>151</sup> and Ser<sup>153</sup> are not known as potential phosphorylation sites in the ICL2 of the GnRHR (18). The data point to an alternative, possibly novel role for the Ser/Thr residues of the ICL2 of type I GnRHR as a core of an inhibitory domain in which Ser<sup>153</sup> and to a lesser degree Ser<sup>151</sup> are key residues.
GnRH Does Not Induce Receptor Phosphorylation in αT3-1 Cells—To corroborate this proposal further, we examined whether the GnRHR undergoes phosphorylation after a GnRH challenge. After overnight serum starvation, αT3-1 cells were labeled for 4 h at 37°C in P3-free DMEM containing 150 μCi/ml 32P, and were washed. GnRH was added for various times, and cell extracts were resolved by SDS-PAGE and visualized using a PhosphorImager as described for the AT1 angiotensin receptor (18). GnRH has no consistent and reproducible effect on receptor phosphorylation.2 Our data are in agreement with Willars et al. (19).

Characterization of the Mutated GnRH Receptors in COS-1 Cells—To analyze further the role of ICL2 and specifically the role of Ser153 in GnRH receptor activation, we prepared two GnRHR mutants. In one of the mutants (ICL2-Ala), two Ser residues (Ser151 and Ser153) and one Thr (Thr142) were mutated to Ala, and in the other mutant only Ser153 was mutated to Ala (S153A). In preliminary studies, we checked whether we could obtain similar binding kinetics in transient transfection of COS-1 cells with the WT GnRH receptor as compared with αT3-1 cells. The results showed similar $K_d$ values for the αT3-1, which expresses the native receptor, and the GnRHR-transfected COS-1 cells ($K_d$ 0.14 ± 0.05 and 0.23 ± 0.04 nM, respectively). A negative control consisting of cells transfected with the vector alone did not exhibit any binding (not shown). We then examined the binding properties of the WT GnRHR and the two mutants ICL2-Ala (S151A, S153A, and T142A) and S153A in the transfected COS-1 cells. Binding competition studies with $^{125}$I-GnRH-A revealed unchanged affinity between the WT and the mutant GnRHRs (Fig. 7A and Table II). Similarly, no significant differences were observed in the expression levels of the HA-FLAG WT and the mutated GnRHR in membranes from transfected COS-1 cells (not shown). To rule out the possibility that receptor activation can differ because of differences in the rate of receptor internalization, cells were transfected with WT or mutated GnRHRs, incubated with $^{125}$I-GnRHA on ice, and then transferred to 37°C to allow internalization. An acid wash was used to remove cell surface-bound ligand, and internalized ligand was measured after solubilizing the cells. As seen in Fig. 7B, agonist-induced internalization was similar for both the WT and the mutated receptors.

2 R. D. Smith, Z. Naor, and K. J. Catt, unpublished observations.
was determined. As shown in Fig. 8A, addition of GnRH to the WT-transfected cells resulted in a 5-fold increase in InsP formation, which reached a peak at 60 min with a t½ of 16 min. On the other hand, stimulation of GnRH in the ICL2-Ala- and S153A-transfected cells also resulted in a 5-fold increase in InsP formation. The peak response was reached already at 30 min, and a remarkable reduction of t½ to 2.5 min was found. Hence, the rate of GnRH-induced elevation of InsP formation was 6.5-fold higher, particularly in the first 10 min in the cells that were transfected with the mutated receptors as compared with the cells with the WT receptor. However, WT and the mutated receptor had similar maximal InsP levels. To assess the role of Ser153 in GnRH-induced ERK1/2 activation, COS-1 cells were transfected with the WT and the mutants of the GnRHR (ICL2-Ala and S153A), and ERK1/2 activation was determined (Fig. 8B). Both mutants enhanced ERK1/2 activation by GnRH. The results support our proposal that the GnRHR harbors inhibitory domains within the ICL2 and that Ser153 plays a key role in this domain.

**DISCUSSION**

Signal termination, receptor desensitization and re-sensitization, and down-regulation are regulated processes mediated by covalent modifications, association with intracellular proteins, internalization, and trafficking of activated GPCRs (1, 2). Rapid homologous desensitization often involves partial or complete uncoupling of the receptors from the effector proteins, which may occur within seconds to minutes of agonist occupancy (1, 2). Considerable evidence has implicated ICL2 and ICL3, as well as the membrane-proximal region of the C terminus of several GPCRs, as involved in G-protein coupling and determination of signal specificity (20, 21). Phosphorylation of GPCR by serine/threonine protein kinases, predominantly through GRKs on phosphorylation sites localized in the C-terminal tail and ICL3, facilitates the binding of arrestin to intracellular domains of GPCRs (1, 2, 22). Arrestin binding induces uncoupling from the G-proteins and facilitates receptor internalization via components of the clathrin endocytic apparatus culminating in receptors being recycled to the cell surface or proteolytically degraded in lysosomes (1, 2, 5, 23, 24).

Another level of receptor regulation is maintained by members of the regulators of G-protein signaling (RGS), a large family of proteins that modulate G-protein activity. RGS proteins interact directly with active Go subunits to accelerate their intrinsic GTPase activity and to limit their half-life, hence curtailing or terminating their activity (25). It was shown recently that RGS2 binds directly to the M1 muscarinic acetylcholine receptor ICL3 to modulate Gq/11 signaling (26). Two family members, RGS3 and RGS10, have been implicated in the regulation of GnRH receptor coupling (27–29), and the C-terminal tails of nonmammalian GnRH receptors may be sites for interactions with RGS10, although the nature of this interaction is unclear (27). Hence, C-terminal tail phosphorylation and modulation by RGSs are now regarded as the main mechanisms leading to desensitization and/or signal termination for GPCRs.

In addition there is precedence for involvement of accessory proteins such as arrestin, GRKs, Src homology 2 domain-containing proteins, small GTP-binding proteins, polyproline-binding proteins, receptor activity-modifying proteins, and members of the scaffolding family of proteins such as PDZ domain-containing proteins in the regulation of GPCR signaling in general. However, the requisite sequence structural motifs in the GnRHR responsible for such interactions are largely unknown (17).

We noticed that the rate of production of InsP stimulated by GnRH in the first 10 min was 10-fold higher than in the following 10–60 min of incubation (1.1- and 0.1-fold/min, respectively). This could result potentially from a receptor phosphorylation that was followed by desensitization. Alternatively, binding of an inhibitory accessory binding partner to the GnRHR could also have triggered the decline in receptor activity. We have therefore utilized synthetic peptides corresponding to the ICLs of the GnRHR to shed light on the mechanisms of signal termination. Synthetic peptides have been shown to modulate receptor and G-protein activities in numerous systems, including the rhodopsin, β- and α2-adrenergic, muscarinic, and dopamine D2 receptors (8, 30, 31). Hence, such peptides may be used to further our understanding of the structure-function relationship of the GnRHR. Although synthetic peptides are usually used in cell-free systems (9), we decided to use cell permeabilization for delivery of the peptides (14). The synthetic peptides had no effect on the basal InsP level, suggesting that activation of Gi by the GnRHR (12, 32) involves structural determinants from more than one ICL. Indeed, Gq activation by the agonist-occupied GnRHR is...
thought to involve determinants in ICL2 (Pro\textsuperscript{146} and Leu\textsuperscript{147}) and ICL3 (Arg\textsuperscript{240}, Val\textsuperscript{241}, Leu\textsuperscript{242}, Arg\textsuperscript{260}, Ala\textsuperscript{261}, and Arg\textsuperscript{262}) (17). Although the peptides had no effect on basal InsP formation, the ICL2 peptide of the GnRHR potentiated GnRH-induced InsP production. A smaller effect was also observed for the ICL3-derived peptides. Because ICL1 and the "mutated" analogs of ICL2 had no effect, the results indicate that the effect exerted by the other peptides was specific. Binding studies showed that introduction of the peptides into αT3-1 cells had no significant effect on GnRH binding.

Conventional GPCR uncoupling and desensitization is mediated through GRK and/or protein kinase A and PKC phosphorylation of the C-terminal tail and ICL3. For GnRHR, we contemplated that the ICLs substitute for the C-terminal tail for this mechanism. If this were the case, the synthetic ICL peptides would act as alternative substrates and protect receptor ICLs from phosphorylation (i.e. as a decoy). However, as mentioned above, mammalian type I GnRHR does not undergo rapid agonist-dependent phosphorylation and desensitization (5), making it unlikely that the peptides would protect the receptor from phosphorylation-dependent desensitization. Indeed, previous studies (19) have shown that the GnRHR is not phosphorylated upon agonist stimulation; a finding confirmed in our current studies. Moreover, GnRHR internalization is independent of β-arrestin (5). It therefore appears that the peptides are alternative targets for other intracellular proteins, which bind to and modulate Gq-coupling to GnRHR. Potential candidates are RGSs, Src homology 2 domain-containing proteins, polypeptide-binding proteins, receptor activity-modifying proteins, and members of the PDZ domain proteins, which are all known to regulate GPCR signaling (17). Therefore, the exogenously added ICL peptides most likely have served as decoy peptides and protected against interaction of the inhibitory proteins with ICL receptor sites.

We therefore suggest that the synthetic peptides mimic receptor ICL domains that are targets for inhibitory proteins, and thus enable further studies to characterize those domains and the accessory proteins that form a complex during receptor activation leading to signal termination. If this is correct, residues of ICL2 and ICL3 are involved in the regulation of receptor activation. Similar results were obtained for the luteinizing hormone receptor, where a synthetic peptide corresponding to the entire ICL3 reversed desensitization of adenyl cyclase activity (33).

Because introduction of ICL2 into permeabilized αT3-1 cells had the most significant potentiating effect on GnRH-induced InsP production, we decided to investigate the residues responsible for the effect. Ser/Thr phosphorylation of GPCRs is one of the most prevalent events in receptor regulation. Three Ser residues (Ser\textsuperscript{140}, Ser\textsuperscript{151}, and Ser\textsuperscript{153}) and one Thr residue (Thr\textsuperscript{144}) are present in ICL2, suggesting that phosphorylation of these residues may be responsible for the ICL2 effect. Hence, to elucidate the role of the Ser/Thr residues of ICL2 in GnRHR regulation, we synthesized mutated peptides in which all the Ser and Thr residues of ICL2, or only one of them, were mutated to Ala or Glu. The stimulatory effect of ICL2 on GnRH-induced InsP formation was abolished when the peptides, in which all the Ser or Thr residues were mutated to Ala or Glu (ICL2-Ala, ICL2-Glu), were inserted to the permeabilized αT3-1 cells, implicating Ser and Thr residues of ICL2 in the protective effect. When the peptides with single substitution of Ser/Thr were inserted to the cells, the two peptides in which Ser\textsuperscript{153} was replaced by Ala or Glu (ICL2-S153A and ICL2-S153E, respectively) did not show the increase in GnRH-induced InsP elevation observed for the ICL2-WT peptide. These results indicate that Ser\textsuperscript{153}, and to a lesser extent Ser\textsuperscript{151}, are important residues in the protective effect of the ICL2 peptide. Therefore, the peptides, in which Ser\textsuperscript{153} was replaced by Ala or Glu, failed to serve as decoy peptides. We had expected that the role of Ser\textsuperscript{153} be as a phosphorylation target, which results in receptor desensitization. The failure of the Ala\textsuperscript{153} to enhance GnRH-stimulated InsP production supports this notion. However, the phosphomimetic Glu\textsuperscript{153} peptide would then be expected to compete effectively for desensitizing proteins that bind to phosphorylated Ser\textsuperscript{153} and enhance stimulation of InsP production as well as, or better than, the WT receptor. Thus, the failure Glu\textsuperscript{153} to enhance GnRH-stimulated InsP production suggests that the intracellular proteins involved in desensitization of GnRHR must bind to a domain that involves Ser\textsuperscript{153} or is configured by Ser\textsuperscript{153} in an unphosphorylated state. This is supported by Willars et al. (19), and our demonstration that GnRHR is not phosphorylated by GnRH.

To confirm further the role of ICL2, and in particular that of Ser\textsuperscript{153} in GnRH activation and signal termination, we transfected COS-1 cells with the WT and two mutants, ICL2-Ala (in which Ser\textsuperscript{151}, Ser\textsuperscript{153}, and Thr\textsuperscript{142} were mutated to Ala) and S153A, and followed agonist-dependent receptor activation. First, a good agreement was noticed between the $K_d$ values for GnRH binding in the αT3-1, which expresses the native receptor, and the GnRHR-expressing COS-1 cells. Second, binding studies with 125I-GnRHR revealed similar parameters between WT and the mutant GnRHRs expressed in the COS-1 cells. Also, no significant differences were observed in the expression levels of the HA-FLAG WT and the mutated GnRHR in membranes from transfected COS-1 cells, and agonist-induced internalization of the GnRHR was similar for both the WT and the mutated receptors.

GnRH stimulated a 5-fold increase in InsP production in WT GnRHR-expressing COS-1 cells, which reached a peak at 60 min with a $t_{\text{1/2}}$ of 16 min. Although a 5-fold increase in InsP production was also observed in the two mutated GnRHRs, the peak response however was already reached at 30 min, and a 6.5-fold reduction of $t_{\text{1/2}}$ to 2.5 min was observed. In addition, ERK activation by GnRH was enhanced in the two mutated GnRHRs. The results support the notion that the GnRHR harbors inhibitory domains within the ICL2 and that Ser\textsuperscript{153} plays a key role in this domain. Nevertheless, a basic difference was observed between the results obtained with the exogenously added peptides of the ICLs in αT3-1 cells and the transfection studies with the WT and the mutants in COS-1 cells. Although in αT3-1 cells the peptides enhanced the maximal response of GnRH-induced InsP formation at 30 min, the mutants had no such effect in the transfected COS-1 cells but had a remarkable effect on the rate of the response. We therefore assume that the inhibitory domains in ICL2 and Ser\textsuperscript{153} manifest themselves differently in different cell type backgrounds because of differences in the intracellular protein milieu.

The ICL2 of the GnRHR was reported in several studies to be involved in signal propagation and G-protein selectivity (17). Although mutations in Ser\textsuperscript{140} resulted in an impaired internalization process (34), mutation of Arg\textsuperscript{139} (part of the DRY motif) to Gln significantly reduced InsP production but did not affect internalization (35). Mutation of another conserved residue, Leu\textsuperscript{147}, to Ala or Asp, also impaired InsP formation (34). Coexpression of a WT GnRHR with a truncated form of the GnRHR, which lacks one-third of the C-terminal region, including ICL3, significantly impaired the signaling ability of the receptor, probably due to interactions of the wild type receptor with the truncated form (36). Exploration of the structural characteristics of ICL2 of the GnRHR using the computational method of conformational memories showed that the wild type ICL2 loop has
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accessible states that can interact with ICL3 (37). However, mutation of the conserved sequence TRPLA of ICL2 to a more constrained TPPLA sequence prevented most of the conformational states of the Pro-Pro mutant from interacting with ICL3. Mutagenesis of Arg in the TRPLA motif to Pro markedly reduced the receptor efficiency, suggesting that ICL2-ICL3 interaction is necessary for efficient G-protein coupling. Collectively, the above studies show that ICL2 is necessary for proper coupling of GnRHR to G<sub>q</sub>. We therefore propose that binding of ICL2 to an inhibitory accessory protein, such as an RGS family member, results in the disruption of GnRHR-G<sub>q</sub> interaction and signal termination. Our results identify Ser<sup>153</sup> as a core in this binding pocket, and we are currently exploring a proteomics approach to identify putative ICL2-binding proteins using WT and mutated ICL2 domains that may be involved in signal termination.

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