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Biochemical Pharmacology

Biochemical Pharmacology 62 (2001) 329–334
Short communication

Critical implication of transmembrane Phe310, possibly in conjunction with Trp279, in the rat gonadotropin-releasing hormone receptor activation

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Received 9 November 2000; accepted 9 April 2001

Abstract

The GnRH-R belongs to the superfamily of heptahelical GPCRs. A three-dimensional model of GnRH binding to its receptor predicted that Trp3 was the most deeply buried residue, potentially allowing it to interact with both Trp279, a highly conserved residue in the TMH 6 of GPCRs, and Phe310, present essentially in TMH 7 of GnRH-Rs. Replacement of Phe310 with Leu, the most common positional residue in GPCRs, induced a slightly decreased B_{max} (1.6-fold) and affinity (3.8-fold); in addition, IP production was completely abolished. Similarly, replacement of Trp279 with Ser depressed the B_{max} by 5.2-fold, the affinity by 2.3-fold, and totally abrogated IP production. The effect of the double mutation was not additive on binding, since the B_{max} was reduced to the level of the Phe310Leu mutant, although the K_d was restored to a value not significantly different from that of the wild-type. The double mutant was also unable to induce IP production. Unexpectedly, no influence of any single or double substitution was noted on receptor internalization. These data provide evidence for the crucial role of Phe310, possibly in conjunction with Trp279, on GnRH transduction and suggest that the conformation for phospholipase C activation may not be required for GnRH-R internalization.

Keywords: GnRH receptor; Three-dimensional modeling; GnRH binding; Receptor activation and internalization; Aromatic residues; Transmembrane helix

1. Introduction

The mammalian GnRH-R is a member of the rhodopsin-like GPCR family. It shares with all GPCRs a common protein structure which includes the presence of seven putative transmembrane α-helices, which contribute to the conformation of the ligand-binding pocket, connected by extra- and intracellular loops functionally crucial for ligand binding and signal transduction, respectively [1]. This receptor also exhibits unique structural features such as the lack of a cytoplasmic tail known to impart in many other GPCRs important regulatory features [2].

Because of the crucial role of GnRH and its receptor in reproductive physiology [3,4], delineation of the precise contact sites between the counterparts is critical for determining the molecular mechanisms underlying receptor activation as well as developing both novel peptide and non-peptide GnRH analogs. The most recent data indicate that the interaction of GnRH with its receptor involves extracellular as well as TMH domains, in a manner consistent with earlier structure–activity studies that demonstrated that both the N- and C-terminal regions of GnRH are important for ligand binding and signal transduction [5]. Taking into consideration all the experimental data related to GnRH/GnRH-R interaction [6–8], we have defined a three-dimensional (3-D) model of the complex between GnRH and its rat receptor. From this model, Trp3 of GnRH was predicted to penetrate into the transmembrane core about 20 Å from the surface of the membrane, interacting with TMH 6 Trp279 [9]. With further refinement of our model, the potential of the Trp3 side chain of GnRH to intercalate between the indole moiety of Trp279 in TMH 6 and the phenyl

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Abbreviations: GnRH, gonadotropin-releasing hormone (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2); GnRH-R, GnRH receptor; IP, inositol phosphate; TMH, transmembrane helix; GPCR, G protein-coupled receptor; and CHO, Chinese hamster ovary.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00675-X

moiety of Phe310 in TMH 7 was revealed, strongly suggesting an interaction between both amino acid residues. To assess this possibility, we examined the effects of mutating Phe310, either alone or in combination with Trp279, on ligand binding, internalization, and IP production. Our results are consistent with the major role of Phe310 on GnRH-R activation, possibly in conjunction with Trp279, and also suggest that the GnRH-R is internalized through a process independent of the activation of phospholipase C (PLC).

2. Materials and methods

A 1.038-kb rat GnRH-R cDNA in pUC19 [10] was used for the construction of wild-type and mutant receptors. Site-directed mutations were made using an *in vitro* mutagenesis method (Takara/Amersham) as described previously [9]. The sequence of the 22-mer mutagenic primer for Phe310 was 5′-TTCTTTCTC**C**TTGCTTTTCTAA-3′; and at the underlined base, codon TTT (Phe) was replaced with CTT (Leu). For Trp279, the 22-mer mutagenic primer was 5′-GTCATCTGCT**C**GGACTCCCTAC-3′ and, at the underlined base, codon TGG (Trp) was altered to TCG (Ser). NheI/XhoI restriction sites (Eurogentec) were created by polymerase chain reaction (PCR) with Vent@$_R$ DNA polymerase (New England BioLabs) in order to subclone into pGEM-T (Promega). The sequences were confirmed by automated DNA sequencing (Li-Cor, MWG-Biotech). The cDNA was then digested and ligated into the expression vector pMSGCAT (Pharmacia Biotech) using the NheI/XhoI restriction sites. For transfection and expression, CHO-K1 cells, routinely maintained at 37° in Ham-F12 medium supplemented with 10% new-born calf serum containing 100 μg/mL of gentaline, were seeded in 6-well plates at a density of 9×10^4 cells per well. Transfection was performed at 60–70% confluence, in 1.2-mL serum-free OPTI-MEM medium per well with 1.2 μg wild-type or mutant plasmid DNA and 24 μg lipofectamine (GIBCO BRL Life Technologies Inc.). Five hours later, the medium was changed and cells were cultured for 60 hr to allow optimal expression of receptors before ligand binding and functional assays. Saturation binding assays were carried out on transfected cells incubated at 25° for 75 min in Ham-F12 as previously described [9], using as the ligand a radioiodinated GnRH analogue [His^5-DTyr^6]GnRH (specific radioactivity, 100 μCi/μg). Internalization assays were performed as described [9] by classic acid-wash (in 50 mM acetic acid and 150 mM NaCl, pH 2.8), removal of extracellular receptor-associated ligand, and solubilization of radioactive ligand retained by cells with NaOH/SDS. Non-specific binding for each time point was determined in the presence of cold agonist excess. IP production was assayed by measuring radioactive IPs isolated by extraction and separation on Dowex ion exchange columns from GnRH-R-expressing CHO cells previously loaded with 6 μCi/ml myo-[2-^{3}H]inositol (Amersham) for 16–18 hr in an inositol and serum-free medium containing 20 mM LiCl, and further incubated for 1 hr in the absence or presence of 10^{-7} M GnRH [9]. Statistical significance was determined by unpaired two-tailed *t*-test.

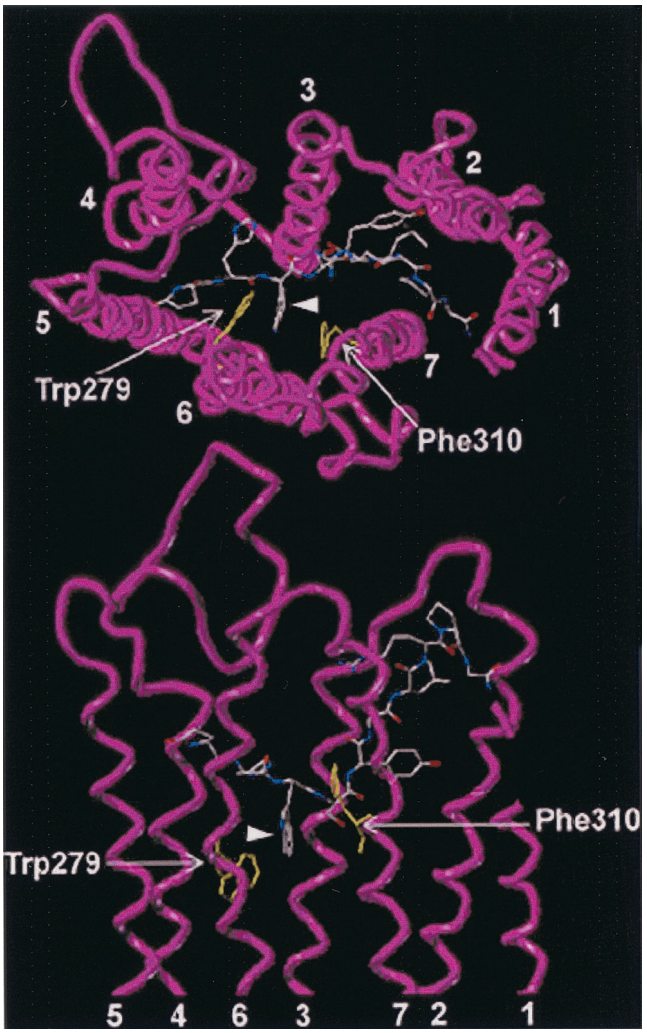

Fig. 1. Model of GnRH docked in its receptor, pointing out the position of Trp3 sandwiched between TMH 6 Trp279 and TMH 7 Phe310. Top: view from the extracellular side of the cell; bottom: transversal side view of the extracellular half. The GnRH-R backbone is shown in magenta, and the Trp279 and Phe310 residues are indicated in yellow. GnRH is colored by atom types (white for carbon, blue for nitrogen, red for oxygen). GnRH Trp3 is annotated by an arrowhead.

The 3-D model of the rat GnRH-R hosting GnRH was constructed using the procedure already extensively described and previously validated [9,11].

3. Results and discussion

The 3-D model of the GnRH docked in its receptor (Fig. 1) was established based on our previous study [9] taking into account general data available on GPCRs and on the GnRH-R in particular [6,11–15]. The general distribution of

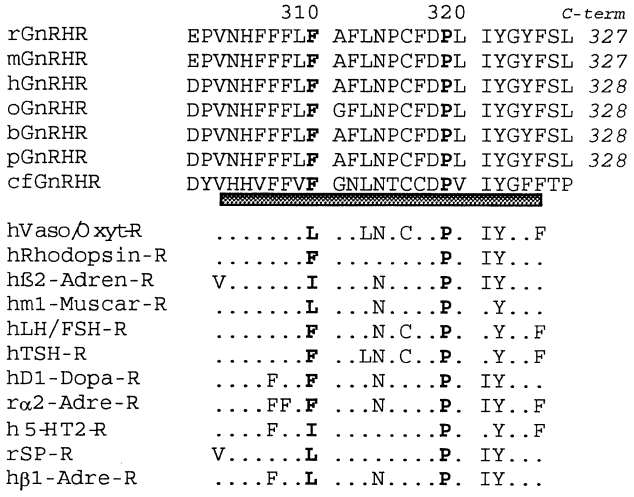

Fig. 2. Alignment of TMH 7 regions of rat (r), mouse (m), human (h), ovine (o), bovine (b), porcine (p), and catfish (cf) GnRH-R primary sequence with representative corresponding sequences of various human (h) or rat (r) GPCRs. Conserved Phe and Pro residues are bolted, and the putative TMH region is underlined. Vaso/Oxyt-R: vasopressin/oxytocin receptor; rhodopsin-R: rhodopsin receptor; β2-Adren-R: β2-adrenergic receptor; m1-Muscar-R: m1 muscarinic receptor; LH/FSH-R: luteinizing hormone and follicle-stimulating hormone receptors; TSH-R: thyroid-stimulating hormone receptor; D1-Dopa-R: D1 dopamine receptor; α2a-Adre-R: α2 adrenergic receptor; 5-HT2-R: 5-hydroxytryptamine (serotonin) receptor; SP-R: substance P receptor; β1-Adre-R: β1 adrenergic receptor.

the decapeptidic neurohormone within the receptor pocket on its side view (Fig. 1, bottom) shows the pyroGlu1 lying at the base of the central receptor cleft in the neighborhood of TMH 5 and the terminal Gly10 carboxamide interacting with Asn102 at top of TMH 2 [8]. His2 may form hydrogen bonds with Lys121 of TMH 3 [6] and Arg8 with Glu301 [7] whereas, according to our model, Trp3 was the most deeply buried residue, located about 20 Å inside the transmembrane core, representing one-third of the membrane depth [9]. This conformation of GnRH, which notably differed from that of the GnRH in solution [16], brings the location of achiral Gly6 near the surface of the binding pocket rather than outside as suggested by others, and thus in a position to interact with another molecule or carry an extended radical [17]. In the present study, we found that further refinement of our model revealed the GnRH Trp3 side chain perfectly sandwiched between Trp279 in TMH 6 and Phe310 in TMH 7 (Fig. 1, top and bottom), implying its possible involvement in π stacking. Interestingly, Trp279 and Phe310 are present in mammalian as well as non-mammalian GnRH-R. Moreover, Trp279 is highly conserved in other GPCRs with the major exception of odorant receptors and glycoprotein hormone receptors, while Phe310 is conserved in certain GPCRs such as human α-adrenergic and glycoprotein hormone receptors, rhodopsins, and opsins (Fig. 2), but in the majority of other GPCRs is mainly replaced by non-aromatic Leu or Ile residues [18].

To further validate our model and examine the respective roles of Phe310 and/or Trp279 in receptor function, these residues were mutated to delete the aromatic side chains. Since the majority of GPCRs contain non-aromatic Leu (or Ile) residues in place of Phe at this position (Fig. 2), we substituted Phe310 with Leu, eliminating its aromatic character but still maintaining the bulkiness of the residue in the GnRH-R. As shown in Table 1, we found that the single Phe310Leu mutation only slightly affected ligand binding compared to wild-type (K_d = 3.95 ± 0.08 vs 1.02 ± 0.10 nM; B_{max} = 335 ± 5 vs 543 ± 13 fmol/mg protein). However, this mutation impaired signal transduction since no IP production could be detected regardless of the concentration of GnRH used (in the range 10^{-10} M to 10^{-7} M), thus providing dose–response curves not different from basal. Table 1 shows the IP response for the wild-type GnRH-R at 10^{-6} M GnRH (EC50 = 2.5 ± 0.4 nM) while still showing no response for the mutant receptors. This result contrasts with the absence of any detectable influence on the time course and degree of internalization (Fig. 3). With the exception of a more severe consequence in term of B_{max}, quite similar conclusions can be drawn from mutating Trp279 residue with Ser, which lacks an indole moiety (Table 1 and Fig. 3). Both these mutant receptors are properly expressed and co-localized with a fluorescent membrane marker (Alexa 594 ConA), as evidenced by the fusion in frame with the green fluorescent protein (data not shown).

At this stage, we considered that if the two loci were independent in their contribution to a measured property, the effect of a mutation at either locus should be additive.

Table 1
Characteristics of GnRH binding and IP production in CHO-K1 cells transfected with wild-type GnRH-R or GnRH-R mutated on Phe310 and/or Trp279

GnRH-R construct	Receptor Binding		IP production (cpm)	
	K_d (nM ± SEM)	B_{max} (fmol/mg prot ± SEM)	Vehicle	10^{-6}M GnRH
Wild-type	1.02 ± 0.10	543 ± 13	564.9 ± 8.4	6250.9 ± 64
F310L	3.95 ± 0.08	335 ± 5	581.4 ± 12.3	562.4 ± 7.3
W279S	2.43 ± 0.11	103 ± 7	535.3 ± 11.0	520.6 ± 9.1
[W279S/F310L]	1.16 ± 0.03	324 ± 12	557.3 ± 13.4	549.9 ± 7.8

Receptor binding assays were performed using ^{125}I-[His^5-DTyr^6]GnRH. K_d and B_{max} were calculated from saturation experiments. IP production was measured in transfected cells labelled with [^{3}H]myo-inositol after incubation for 1 hr in the absence or presence of 10^{-6} M GnRH. Values are the means ± SEM of 8 individual experiments.

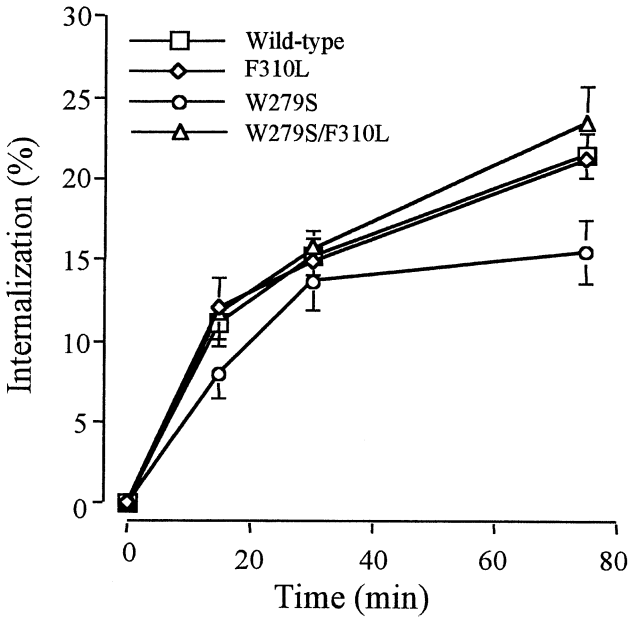

Fig. 3. Agonist-induced internalization in GnRH-R-transfected CHO-K1 cells. Cells expressing the mutant and native GnRH-R were incubated with ^{125}I-[His^5-DTyr^6]GnRH for the indicated time at 25°. The extracellular ligand was removed by acid wash and the radioactivity determined. Internalized radioactivity was measured after solubilizing the cells. Non-specific binding for each time was determined in the presence of an excess of unlabeled agonist. After subtraction of non-specific binding, the internalized radioactivity was expressed as a percentage of total specific binding at that time interval. All time points were performed in triplicate and are means ± SEM of at least 3 independent experiments.

Thus, we constructed a double mutant receptor composed of the two mutations: Phe310Leu and Trp279Ser. Interestingly, the [Phe310Leu/Trp279Ser] mutation restored affinity with a K_d not significantly different from that of the wild-type receptor (K_d = 1.16 ± 0.03 nM). In addition, the B_{max} was quite similar to that displayed by the single Phe310Leu mutant (B_{max} = 324 ± 12 fmol/mg protein vs 335 ± 5 fmol/mg protein), i.e. much higher than the Trp279Ser mutant (Table 1). The internalization was, however, again unaffected by these both mutations (Fig. 3).

The fact that all mutants studied in this work were deficient in G-protein coupling/PLC activation but unimpaired in receptor internalization is particularly intriguing. This suggests a dissociation between these two processes, whereas it is generally accepted that agonist-induced internalization requires an active receptor conformation. The possibility that mutants produced IP in response to GnRH but at levels too low to be detected is in our opinion improbable, although it is difficult to be excluded totally. Indeed, even if the mutant response were very weak, we should be able to detect it under GnRH concentrations as high as 10^{-6} M, while responses to concentrations as low as 10^{-10} M could be routinely detected with the wild-type [9,19,20]. In addition, the modest changes in receptor number and affinity appear insufficient to explain the loss of IP responses particularly in the Phe310Leu and the double [Trp279Ser/Phe310Leu] mutants.

Other examples of a dissociation between PLC activation and internalization in GPCRs are provided by the β2-adrenergic and the angiotensin 1A receptors [21–23]. In contrast to these receptors, the mammalian GnRH-R has the unique peculiarity to lack the C-terminal intracellular tail that is crucial for GRK-induced phosphorylation and β-arrestin binding, a process that would represent an early step in agonist-induced internalization [24,25]. Indeed, the mammalian GnRH-R is not phosphorylated in an agonist-dependent manner [26] and internalizes via a GRK- and β-arrestin-independent process [26,27]. In addition, internalization may involve the contribution of specific residues located in the second intracellular loop as well as in TMH7 [28,29]. Thus, in addition to the data from the literature suggesting that GnRH-R internalization occurs through an atypical process as compared to other GPCRs, our results argue in favor of distinct GnRH-induced conformational changes required for GnRH-R internalization and PLC activation.

These data also support the hypothesis that Phe310 and Trp279 are located in the same region and contribute to a network of interactions. The fact that the double mutant [Phe310Leu/Trp279Ser] is recognized by GnRH with the same affinity as the wild-type implies that the mutation of these two residues induces a conformational change that compensates for the dramatic conformational change induced by each mutation itself. Alternatively, there may exist other anchoring points in GnRH contributing predominantly to the overall affinity. Clearly, Phe310 and Trp279 are crucial in GnRH-induced receptor activation, since all of the studied mutations (single as well as double) resulted in the total abolition of IP production in response to GnRH.

The same Trp corresponding to Trp279 in GnRH-R has been shown to play a pivotal role in a number of receptors including rhodopsin, cholecystokinin B, and angiotensin A1 receptors [30–32]. However, this would be the first time that a functional implication of Phe310 has been described, specifically for GnRH-R. For the moment, it is difficult to speculate on the mechanisms by which these residues may intervene in the GnRH-R activation, although in the β_2-adrenergic and 5-hydroxytryptamine (serotonin) 2_A receptors as well as rhodopsin, movement of TMH 6 has been associated with receptor activation [33–36]. Whether interaction of Trp279 or Phe310 may induce similar changes has not been established so far. We can postulate according to Javitch [37] that the ligand binding may induce movements of these residues, resulting in a rotational translational movement about the conserved proline kink. It would be possible to assess this eventuality using Gether's technique, which takes advantage of the sensitivity of many fluorescent molecules to the polarity of their molecular environment [34].

In conclusion, we have proposed a docking mode for GnRH in its receptor that is compatible with previous experimental data and supports the simultaneous interaction of

GnRH Trp3 with both a Trp279 residue on TMH 6 and the neighboring Phe310 residue on TMH 7. Through mutagenesis, we showed that these two residues are not crucial for ligand binding and internalization but are involved in signal transduction. In addition, the data implicitly suggest that the active GnRH-R conformations required for internalization and PLC activation might be distinct. Further studies are in progress to more precisely examine these aromatic interactions as well as other structural predictions from the 3-D model.

Acknowledgements

The authors wish to express their warmest thanks to Dr. R.P. Millar (Capetown, South Africa and Edinburgh, UK) for kindly providing the [His^5-$DTyr^6$]GnRH, and Drs D. Leiber and S. Harbon, EP1088 CNRS, Orsay for their help in setting up the method for IP measurement. We are indebted to M.Y. Brossas for his help in automated DNA sequencing and Drs J. Treton and Y. Courtois, U-450 Inserm, Paris for kindly giving us free access to the LI-COR DNA sequencer. We gratefully acknowledge the contribution of Ms. M.-C. Chenut for the preparation of the manuscript. This work was supported by grants from the CNRS, the Université Pierre et Marie Curie, and the Association pour la Recherche sur le Cancer (ARC). S.C. was recipient of a grant from ARC.

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