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Glutamate 301 of the Mouse Gonadotropin-releasing Hormone Receptor Confers Specificity for Arginine 8 of Mammalian Gonadotropin-releasing Hormone*

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Colleen A. Flanagan‡§¶, Inga I. Becker‡, James S. Davidson‡, Ian K. Wakefield‡, Wei Zhou‖, Stuart C. Sealfon‖, and Robert P. Millar‡§**

*From the ‡Medical Research Council Regulatory Peptides Research Unit, Department of Chemical Pathology and the §Endocrine Laboratory, Department of Medicine, University of Cape Town Medical School, Observatory, 7925, South Africa and the ‖Fishberg Research Center in Neurobiology and the **Department of Neurology, Mount Sinai School of Medicine, New York, New York 10029*

The Arg residue at position 8 of mammalian GnRH is necessary for high affinity binding to mammalian GnRH receptors. This requirement has been postulated to derive from an electrostatic interaction of Arg^8 with a negatively charged receptor residue. In order to identify such a residue, 8 conserved acidic residues of the mouse GnRH receptor were mutated to isosteric Asn or Gln. Mutant receptors were tested for decreased preference for Arg^8-containing ligands by ligand binding and inositol phosphate production.

One of the mutants, in which the Glu^{301} residue was mutated to Gln, exhibited a 56-fold decrease in apparent affinity for mammalian GnRH. The mutant receptor also exhibited decreased affinity for [Lys^8]GnRH, but its affinity for [Gln^8]GnRH was unchanged compared with the wild type receptor. The apparent affinity of the mutant receptor for the acidic analogue, [Glu^8]GnRH, was increased more than 10-fold. The mutant receptor did not, therefore, distinguish mammalian GnRH from analogues with amino acid substitutions at position 8 as effectively as the wild type receptor. This loss of discrimination was specific for the residue at position 8, because the mutant receptor did distinguish mammalian GnRH from analogues with favorable substitutions at positions 5, 6, and 7. These findings show that Glu^{301} of the GnRH receptor plays a role in receptor recognition of Arg^8 in the ligand and are consistent with an electrostatic interaction between these 2 residues.

The hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH)[1] is the central regulator of reproductive function. The structures of the amino and carboxyl termini of GnRH have been conserved over 500 million years of evolution. The most variable residue is at position 8. In mammalian GnRH this is a positively charged Arg residue, which is necessary for high affinity and specificity of binding to mammalian GnRH receptors (GnRHR). In contrast, GnRHs from most non-mammalian vertebrates contain uncharged residues at position 8 (1). Although these non-mammalian GnRHs are fully active in the animals in which they occur naturally, they show diminished capacity to stimulate release of gonadotropins from mammalian pituitary cells (1) and to stimulate inositol phosphate (IP) production in cells transfected with mammalian GnRHRs (2). Experiments using synthetic GnRH analogues have confirmed the importance of a positively charged amino acid at position 8 for high affinity interaction with mammalian GnRHRs (3–5).

It has been postulated that the Arg^8 side chain may interact directly with the GnRHR via an electrostatic interaction with a negatively charged Asp or Glu residue (6) or with a sialic acid residue in the carbohydrate moiety of this glycoprotein (7). A functional GnRHR was first cloned from the mouse αT3 gonadotroph cell line (8). This and the subsequent cloning of three other mammalian GnRHRs (2, 9–13) have allowed the application of site-directed mutagenesis in identifying amino acid residues which determine the specificity of mammalian GnRHRs for GnRHs which contain Arg at position 8.

The ligand binding sites of heptahelical G-protein-coupled neurotransmitter receptors are contained within the transmembrane helical bundle (14, 15). However, the larger size of peptide hormones suggests that the extracellular loops of their receptors may also participate in ligand binding functions. To test the possibility that the high affinity of mammalian GnRH, which contains Arg^8, is dependent on an acidic residue, we systematically mutated conserved acidic residues in the extracellular and transmembrane domains of the mouse GnRHR. We show here that one of these mutant receptors, the [Gln^{301}]GnRHR, displayed decreased ligand binding affinity for mammalian GnRH, but affinities for GnRH analogues with uncharged residues at position 8 were unchanged or increased.

MATERIALS AND METHODS

Peptides—GnRH, [Gln^8]GnRH (chicken GnRH I), chicken GnRH II ([His^5,Trp^7,Tyr^8]GnRH), [D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]GnRH, [D-Trp^6]-GnRH, [D-Trp^6,Pro^9-NHEt]GnRH, and [D-Trp^6,Gln^8,Pro^9-NHEt]GnRH were prepared by conventional solid-phase methodology and purified by preparative C-18 reversed-phase chromatography. [Glu^8]GnRH and [His^8]GnRH were gifts from R. W. Roeske and [Lys^8]GnRH was a gift from J. Rivier.

Mutagenesis and Transfection—The mouse GnRHR was cloned into pBluescript II SK+ (Stratagene, La Jolla, CA) and site-directed mutagenesis was performed using uracil-containing DNA (16) for [Gln^8]GnRHR,[Gln^{111}]GnRHR,[Asn^{185}]GnRHR,[Asn^{292}]GnRHR,[Gln^{294}]-GnRHR, and [Gln^{301}]GnRHR. For [Gln^{90}]GnRHR and [Asn^{98}]GnRHR, the mouse GnRHR was cloned into the pALTER™-1 vector and mutated using altered sites *in vitro* mutagenesis system (Promega). To confirm mutagenesis, DNA was sequenced manually, using a Sequenase kit

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¶ To whom correspondence should be addressed: MRC Regulatory Peptides Research Unit, Dept. of Chemical Pathology, University of Cape Town Medical School, Observatory, 7925, South Africa. Tel.: 27-21-406-6532; Fax: 27-21-406-6153.

[1] The abbreviations used are: GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; IP, inositol phosphates; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

(U. S. Biochemical Corp.) or by automated sequencer (Bio-Rad). The receptor was subcloned into the expression vector pcDNAI/Amp (Invitrogen Corp., San Diego, CA), and mutation sites were resequenced.

COS-1 cells were transiently transfected with pcDNAI/Amp-GnRHR constructs using a modification of the DEAE-dextran method (17) as described previously (12). 2.5 μg of DNA construct was used per well in 12-well plates for IP assays and 15 μg of DNA/10-cm dish for ligand binding assays. Receptor expression ranged from 150 to 600 fmol/10^6 cells for the wild type GnRHR and from 35 to 150 fmol/10^6 cells for the [Gln^{301}]GnRHR.

IP Production—Transfected cells were labeled overnight with [^{3}H]inositol (2 μCi/ml) and stimulated with GnRH or GnRH analogues for 60 min in the presence of LiCl (10 mM). The reaction was terminated by addition of a perchloric acid solution and phytic acid. After neutralizing with KOH, inositol phosphates were separated on Dowex ion exchange columns and counted (18).

Radioligand Binding Assay—Ligand binding assays with the wild type GnRHR and the screening ligand binding assay were performed as described previously (12). Briefly, transfected COS-1 cells were detached from culture dishes in binding buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 0.1% BSA, fatty acid-free), homogenized with a Dounce homogenizer, and centrifuged at 15,000 × *g* for 30 min at 4 °C. The crude membrane pellet was resuspended in binding buffer and incubated (7.5 × 10^5 cell eq/tube, ~200 fmol of receptor) with 60,000 cpm of ^{125}I-[D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]GnRH (~50 pM) and varying concentrations of unlabeled test peptides in a final volume of 0.5 ml for 60–90 min on ice. The incubation was terminated by the addition of 3 ml of phosphate-buffered saline (PBS, pH 7.5) containing 0.1% BSA and immediate filtration through glass fiber filters (GF/C, Whatman) presoaked in PBS containing 1% BSA. The filters were washed twice with 0.1% BSA-PBS, and the retained radioactivity was counted. Nonspecific binding was estimated in the presence of 10^{-7} M unlabeled [D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]GnRH.

To compensate for the lower total binding of ^{125}I-[D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]GnRH exhibited by the [Gln^{301}]GnRHR, higher concentrations of membranes (1.2 × 10^6 cell eq/tube, ~75 fmol of receptor) were used for subsequent experiments on the mutant receptor. Also, to avoid dissociation of the labeled ligand from the lower affinity receptor, the dilution step at the end of the assay was eliminated and the filters washed four times under vacuum with 0.1% BSA-PBS to remove nonspecifically bound ^{125}I-[D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]GnRH. Increasing amounts of the pcDNAI/Amp-[Gln^{301}]GnRHR construct in the transfection reaction showed that maximal expression of the mutant receptor was achieved with 15 μg of DNA/10-cm dish of cells, the same as with the wild type GnRHR construct. Binding of ^{125}I-[D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]GnRH was maximal after incubation for 75 min and remained stable for a further 75 min. Specific binding ranged from 2004 to 4518 cpm/tube (0.8–1.9 fmol, compared with 11,700 to 20,543 cpm/tube, 4.8–8.6 fmol, with wild type GnRHR), whereas nonspecific binding ranged from 2189 to 3228 cpm/tube (0.9–1.3 fmol).

Data Analysis—Peptide concentrations required to half-maximally inhibit binding of ^{125}I-[D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]GnRH (IC_{50} values) and to stimulate half-maximal IP production (EC_{50} values) were estimated by four-parameter nonlinear curve fitting using Sigmaplot (Jandel Scientific, Corte Madera, CA). Binding assays were performed in triplicate, and IC_{50} values were determined in three to five independent experiments. IC_{50} data in Table I are means ± S.E. of all experiments. Competitive binding curves for some GnRH peptides exhibited slopes which render Hill coefficients not equal to one. For this reason, we have used IC_{50} values as indicators of apparent ligand binding affinity and supported our observations of changes in apparent affinity by measuring ED_{50} values for IP production in response to all GnRH peptides. IP assays were performed in duplicate, and EC_{50} values were determined in two or three independent experiments. EC_{50} data in Table I are the mean ± S.E. of all experiments. For Figs. 3–6, individual IP and binding data points from all experiments were averaged, and curves were drawn using four-parameter nonlinear curve-fitting as above.

RESULTS

Identification of a Mutant GnRHR Which Does Not Discriminate GnRH and [Gln^8]GnRH—We identified 8 acidic amino acid residues (Fig. 1) in the extracellular and superficial transmembrane domains of the mouse GnRHR which are conserved as acidic residues in all of the cloned GnRHRs and which were therefore candidates for interaction with Arg^8 of GnRH. If the

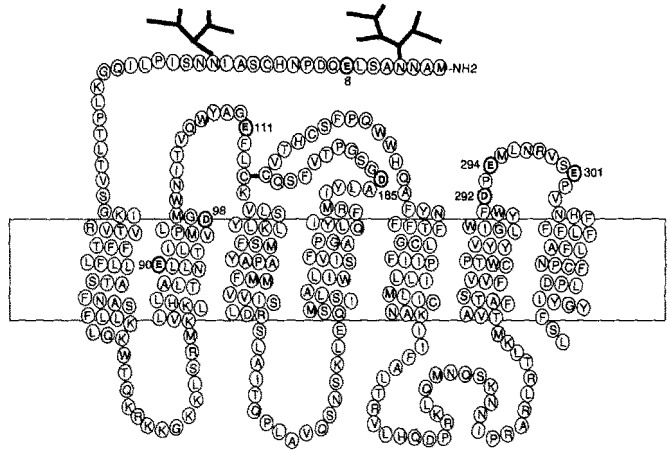

FIG. 1. **The amino acid sequence and proposed secondary structure of the mouse GnRHR.** Conserved acidic residues in the extracellular and superficial transmembrane domains are indicated by their sequence numbers and *bold typeface*.

Arg^8 of GnRH were to interact directly with one of the acidic residues of its receptor, a mutant GnRHR in which this interaction is disrupted would be expected to have low affinity for the ^{125}I-[D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]GnRH used in the receptor binding assay. Because this decreased affinity would result in low specific binding of the labeled GnRH agonist, mutant GnRHRs were first screened for their ability to support GnRH-stimulated IP production. 10^{-8} M GnRH is just sufficient to cause maximal IP production in the wild type GnRHR. A mutant GnRHR with decreased affinity for GnRH, but normal coupling to phospholipase C, should exhibit decreased IP production in response to 10^{-8} M GnRH, but a normal maximal response to 10^{-5} M GnRH.

Systematic substitution of 6 acidic residues, Glu^8, Glu^{90}, Glu^{111}, Asp^{185}, Asp^{292}, and Glu^{294}, with their isosteric amides did not cause large changes in the ability of the mutant GnRHRs to support GnRH-stimulated IP production (Fig. 2). Two GnRHR mutants, [Asn^{98}]GnRHR and [Gln^{301}]GnRHR, demonstrated reduced IP production in response to 10^{-8} M GnRH (Fig. 2). Of these, only the [Gln^{301}]GnRHR demonstrated a full response to 10^{-5} M GnRH (Fig. 2). In a screening ligand binding assay, all mutant GnRHRs except [Asn^{98}]GnRHR exhibited specific binding of ^{125}I-[D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]GnRH (data not shown).

In competitive ligand binding assays the [Gln^{301}]GnRHR showed decreased apparent affinity for GnRH, but the apparent affinity for [Gln^8]GnRH was similar to that of the wild type receptor (Table I; Fig. 3). Both peptides showed low potency in stimulating IP production in COS-1 cells transfected with the [Gln^{301}]GnRHR with very similar dose-response curves (Table I; Fig. 3).

Activities of Other Position 8-substituted GnRHs—GnRH analogues with Lys, His, or Glu substituted for Arg^8 exhibited low apparent affinities for the wild type GnRHR. [Lys^8]GnRH was most potent, whereas the negatively charged [Glu^8]GnRH was least potent, being unable to cause 50% inhibition of binding of the labeled GnRH agonist at concentrations up to 10^{-4} M (Table I; Fig. 4). Apparent affinities of these peptides for the mutant [Gln^{301}]GnRHR were also low. The mutant receptor exhibited 3-fold decreased affinity for the positively charged [Lys^8]GnRH and increased affinities for [His^8]GnRH and for [Glu^8]GnRH (Table I; Fig. 4).

Consistent with the ligand binding results, GnRH analogues with Lys and His residues at position 8 were less potent in stimulating IP production with the [Gln^{301}]GnRHR than with wild type, whereas the acidic [Glu^8]GnRH showed only a small

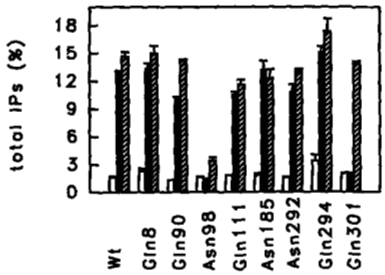

FIG. 2. **Screening assay for GnRH-stimulated IP production in COS-1 cells transfected with GnRHR mutants in which conserved acidic residues were mutated to Asn or Gln.** Transfections were performed as described under "Materials and Methods." Mutant receptors are identified by the name of the new residue (Gln or Asn) and its sequence number. *Wt* is the wild type mouse GnRHR. IP production was measured in the absence of GnRH (*open bars*) and in the presence of 10^{-8} M (*solid bars*) and 10^{-5} M GnRH (*cross-hatched bars*).

reduction in potency (Table I; Fig. 4). [Glu^{8}]GnRH did not stimulate maximal IP production in cells transfected with either wild type or mutant receptors (Fig. 4). Thus, mammalian GnRH and all position 8-substituted GnRH analogues showed low potency in activating the mutant GnRHR (Table I; Fig. 4).

Other GnRH Agonists—Low affinity binding interactions are generally less specific than high affinity interactions. Thus, a mutation which causes general disruption of the configuration of the ligand binding site, rather than eliminating a specific interaction, could generate a low affinity receptor which also does not discriminate different GnRH analogues, as was found for the [Gln^{301}]GnRHR. To test whether this loss of discrimination was specific for modifications of GnRH at position 8, the activities of GnRH analogues which have high affinity due to modifications of other residues were tested. Chicken GnRH II ([His^{5},Trp^{7},Tyr^{8}]GnRH) and two conformationally constrained GnRH superagonists, [D-Ala^{6},*N*-Me-Leu^{7},Pro^{9}-NHEt]GnRH and [D-Trp^{6}]GnRH, showed higher apparent affinity for the [Gln^{301}]GnRHR than did GnRH or analogues containing substitutions only at position 8 (Table I; Fig. 5). These peptides were also more active than GnRH and [Gln^{8}]GnRH in stimulating IP production in cells transfected with the [Gln^{301}]-GnRHR (Table I; Fig. 5). Thus, the [Gln^{301}]GnRHR exhibited high affinity for GnRH agonists containing favorable substitutions at positions 5, 6, 7, and 10 and was able to discriminate between them and GnRH. This indicates specific loss of an interaction which requires Arg^{8}, rather than a generalized decrease in binding affinity.

Superagonists with and without Arg^{8}—Having identified a residue (Glu^{301}) in the GnRHR which determines the specificity of the receptor for Arg^{8} in GnRH, we addressed the question of whether this specificity depended on a direct interaction between the side chains of the Arg^{8} and Glu^{301} residues or on the intramolecular function of Arg^{8} in stabilizing the preferred conformation of GnRH. To do this we attempted to discount the intramolecular role of Arg^{8} by comparing the activities of two high affinity GnRH agonists ([D-Trp^{6},Pro^{9}-NHEt]GnRH and [D-Trp^{6},Gln^{8},Pro^{9}-NHEt]GnRH) in which the preferred conformation is constrained by D-Trp at position 6 and which differ from each other only at position 8. Both peptides exhibited high affinity binding to both wild type and mutant receptors. The Gln^{8}-containing peptide showed higher apparent affinity for the mutant [Gln^{301}]GnRHR than for the wild type receptor (Table I; Fig. 6). The Gln^{8}-containing peptide also exhibited no loss of potency in stimulating IP production in the mutant receptor compared with wild type (Table I; Fig. 6). The rank order of potency of the two peptides was reversed in the [Gln^{301}]GnRHR, with the Gln^{8}-containing peptide more potent than the Arg^{8}-containing peptide in stimulating IP production, although binding affinities were similar for both peptides (Table I; Fig. 6). These results indicate that the mechanism by which the Glu^{301} residue confers specificity for Arg^{8} is more complex than either a simple electrostatic interaction or simple stabilization of ligand conformation.

DISCUSSION

Eight GnRHR mutants were constructed, in which acidic residues in the superficial transmembrane and extracellular domains were exchanged for uncharged hydrophilic amide residues with equivalent side chain lengths. Six mutant GnRHRs exhibited normal GnRH-stimulated IP production in a screening assay, whereas two mutations caused changes in IP production. Mutation of Asp^{98} caused a loss of both agonist ligand binding and GnRH-stimulated IP production, suggesting that this mutation may affect expression or stability of the receptor or disrupt the configuration of the ligand binding site.

The [Gln^{301}]GnRHR mutant had characteristics consistent with loss of specificity for Arg at position 8 of GnRH. It exhibited low apparent affinity for GnRH, similar to the low apparent affinity of the wild type receptor for GnRH analogues which do not contain Arg at position 8. Its apparent affinity for [Gln^{8}]GnRH was unchanged from that of the wild type receptor for this ligand. However, its apparent affinity for [Glu^{8}]GnRH was more than 10-fold higher than that of the wild type receptor. The mutant receptor did not clearly distinguish GnRH from analogues which have substitutions exclusively at position 8. Since low affinity interactions often lack specificity, it might be argued that the [Gln^{301}]GnRHR is simply a low affinity receptor which has lost the ability to discriminate different ligands. However, the [Gln^{301}]GnRHR retained appropriate affinities for GnRH analogues which contain activating substitutions in other positions. Thus, removal of the negatively charged Glu^{301} residue of the GnRHR removed the preference of the receptor for Arg at position 8 of GnRH, but it did not remove the preference of the receptor for ligands with favorable substitutions at positions 5 and 7, as illustrated by chicken GnRH II, nor did it remove the preference for a D-amino acid at position 6. Thus the Glu^{301} residue of the mouse GnRHR determines the ability of the receptor to recognize Arg^{8} in GnRH.

It has been proposed that the Arg^{8} of GnRH participates in a direct ionic interaction with 1 or more negatively charged residues in the receptor, either an amino acid side chain (6) or a polysaccharide sialic acid residue (7). The present results, combined with our recent demonstration that mutation of each of the putative glycosylation sites in the GnRHR does not affect ligand binding affinity,[2] support the involvement of an amino acid side chain, rather than a sialic acid residue, in the interaction of the GnRHR with Arg^{8}.

An electrostatic interaction is also supported by the experiments with [Glu^{8}]GnRH which has a negatively charged residue at position 8. The very low binding affinity ($IC_{50} > 10^{-4}$ M) of the wild type GnRHR for [Glu^{8}]GnRH is consistent with repulsion between the negative charges of Glu^{8} in the peptide and Glu^{301} in the GnRHR. Removing the negative charge of the amino acid at position 301 of the receptor improved the apparent binding affinity of this negatively charged ligand more than 10-fold (IC_{50}, 2.21×10^{-5} M in the [Gln^{301}]GnRHR).

However, the mechanism by which specificity for Arg^{8} is conferred by Glu^{301} appears more complex than a simple electrostatic interaction. Like many peptides, GnRH is highly flexible in solution and exists as an equilibrium mixture of structural conformers. It has been proposed that the side chain of Arg^{8} stabilizes a preferred conformation of GnRH by forming a structural unit of hydrogen bonding with the side chains of His[2]

[2] J. Davidson *et al.*, manuscript in preparation.

TABLE I
Summary of ligand binding and IP production results

Peptide	Wild type GnRHR		[Gln^{301}]GnRHR		Activity index[a]	
	IC_{50}	EC_{50}	IC_{50}	EC_{50}	Binding	IP
	nM		nM			
GnRH	20.9 ± 7.9	1.14 ± 0.02	1180 ± 74	333 ± 80	56.5	292
[Lys^{8}]GnRH	1050 ± 254	86.4 ± 30.0	3290 ± 494	1420 ± 320	3.13	16.4
[Gln^{8}]GnRH	2530 ± 175	42.0 ± 2.5	1870 ± 498	369 ± 67	0.74	8.78
[His^{8}]GnRH	4630 ± 1410	311 ± 140	950 ± 388	1010 ± 410	0.21	3.25
[Glu^{8}]GnRH	$>10^{5}$	1160 ± 180	22100 ± 4410	1750 ± 460	<0.2	1.51
[His^{5},Trp^{7},Tyr^{8}]GnRH (chicken GnRH II)	48 ± 10	5.3 ± 1.6	106 ± 32.2	29.4 ± 9.0	2.21	5.5
GnRHAg[b]	1.50 ± 0.35	0.074 ± 0.013	7.06 ± 2.44	9.90 ± 0.45	6.6	134
[D-Trp^{6}]GnRH	2.08 ± 0.42	0.095 ± 0.032	1.18 ± 0.37	15.9 ± 1.4	0.57	167
[DTrp^{6},Pro^{9}-NHEt]GnRH	1.86 ± 0.26	0.048 ± 0.007	1.38 ± 0.44	3.60 ± 2.47	0.74	75
[D-Trp^{6},Gln^{8},Pro^{9}-NHEt]GnRH	10.1 ± 0.59	0.32 ± 0.22	2.26 ± 0.64	0.33 ± 0.04	0.22	1.03

[a] Activity index, which indicates the apparent decrease in potency of each peptide due to the mutation of Glu^{301}, was calculated as the ratio of the IC_{50} values (binding) or EC_{50} values (IP production) in the [Gln^{301}]GnRHR and the wild type GnRHR. An activity index of 1 indicates identical activity in both receptors.
[b] GnRHAg is [D-Ala^{6},*N*-Me-Leu^{7},Pro^{9}-NHEt]GnRH.

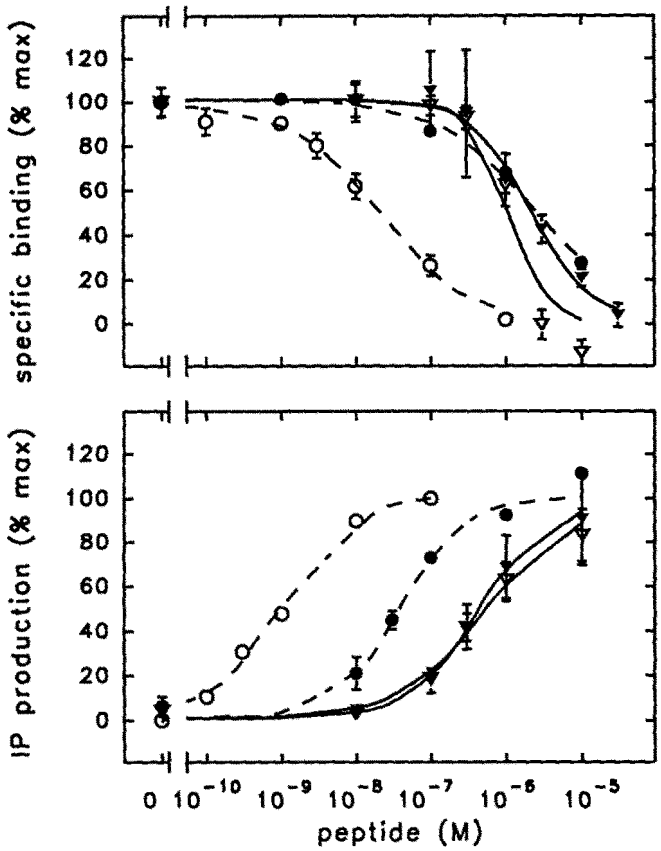

FIG. 3. **GnRH and [Gln^{8}]GnRH ligand binding and IP production in COS-1 cells transfected with wild type GnRHR and [Gln^{301}]GnRHR.** Binding of ^{125}I-[D-Ala^{6},*N*-Me-Leu^{7},Pro^{9}NHEt]GnRH (*top panel*) in the presence of various concentrations of GnRH (○, ▽) and [Gln^{8}]GnRH (●, ▼) to membranes prepared from COS-1 cells transfected with wild type GnRHR (– – – –, ○, ●) or [Gln^{301}]GnRHR (—, ▽, ▼) was measured as described under "Materials and Methods." Data points are the mean ± S.E. of three to five experiments performed in triplicate. IP production (*lower panel*) in response to GnRH (○, ▽) and [Gln^{8}]GnRH (●, ▼) in COS-1 cells transfected with wild type GnRHR (– – – –, ○, ●) and [Gln^{301}]GnRHR (—, ▽, ▼) as described under "Materials and Methods." Data points are the means of two or three experiments performed in duplicate.

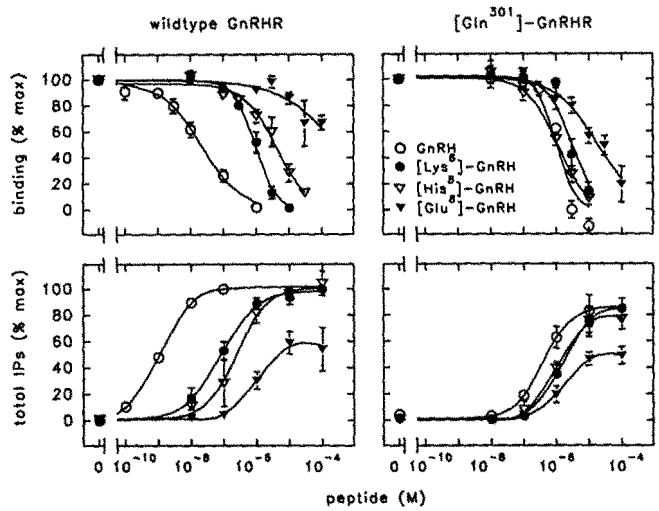

FIG. 4. **Ligand binding and IP production of position 8-substituted GnRH analogues in COS-1 cells transfected with wild type GnRHR and [Gln^{301}]GnRHR.** Binding of ^{125}I-[D-Ala^{6},*N*-Me-Leu^{7},Pro^{9}NHEt]GnRH (*top panels*) and IP production (*lower panels*) in the presence of various concentrations of GnRH, [Lys^{8}]GnRH, [His^{8}]GnRH, and [Glu^{8}]GnRH in COS-1 cells transfected with wild type GnRHR (*left panels*) or [Gln^{301}]GnRHR (*right panels*) were measured as described under "Materials and Methods." Binding data points are the mean ± S.E. of three to five experiments performed in triplicate, and IP data points are the means of two or three experiments performed in duplicate.

and Tyr^{5} (19–21). It has also been shown that although this type of structural unit is formed in GnRH, it is not formed in the neutral [Gln^{8}]GnRH analogue (22). Other studies have indicated that GnRH interacts with its receptor in a folded conformation with a β-turn which involves Gly^{6}. This conformation can be stabilized by substitution of Gly^{6} with a D-amino acid, which increases GnRH activity (23–25). Therefore, if Gly^{6} in GnRH is substituted with D-Trp, the ligand can be constrained in the preferred conformation, independently of whether or not there is Arg at position 8. This constraint makes it possible to distinguish the role of Arg^{8} in interacting with the receptor Glu^{301} residue from its contribution to ligand stabilization. Thus, we compared the apparent binding affinities, in wild type and mutant receptors, of two conformationally constrained GnRH agonists ([D-Trp^{6},Pro^{9}-NHEt]GnRH and [D-Trp^{6},Gln^{8},Pro^{9}-NHEt]GnRH) which are identical except for the substitution of Gln for Arg at position 8. If there were a simple electrostatic interaction between Arg^{8} and Glu^{301}, constrained analogues would be expected to show the same losses of affinity as did the unconstrained peptides. Thus, the affinity of the wild type GnRHR for the constrained analogue containing Gln at position 8 should be 50-fold lower than its affinity for the constrained Arg^{8} analogue. Also the mutant receptor should exhibit similar lower affinities for both constrained peptides. In contrast, if there is no electrostatic interaction between these residues and the higher affinity of GnRH compared with

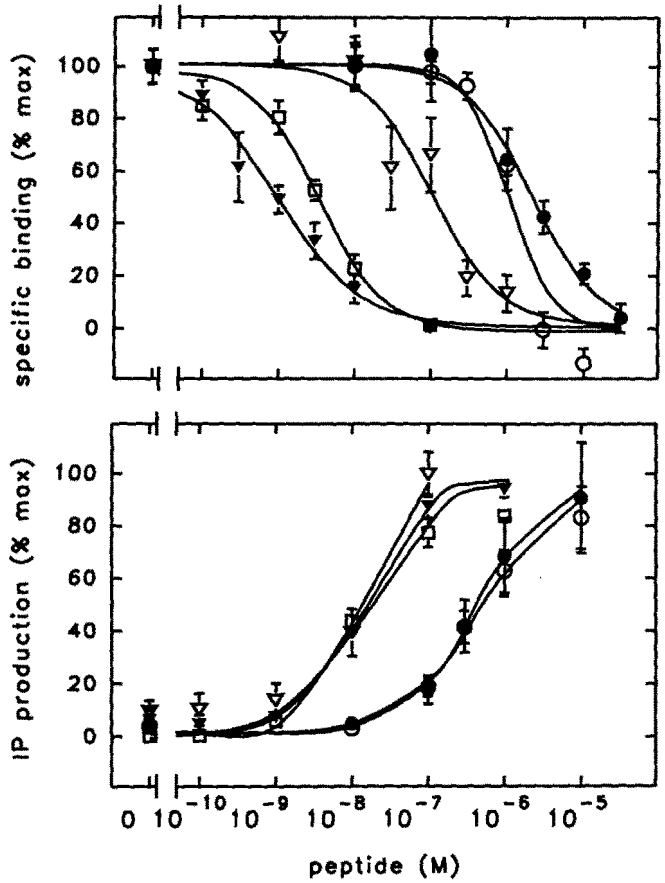

FIG. 5. **The mutant [Gln^{301}]GnRHR exhibits wild type specificity for GnRH analogues with substitutions at positions 5, 6, 7, and 10.** Binding of ^{125}I-[D-Ala^{6},*N*-Me-Leu^{7},Pro^{9}NHEt]GnRH (*top panel*) and IP production (*lower panel*) in the presence of various concentrations of GnRH (○), [Gln^{8}]GnRH (●), chicken GnRH II (▽), [D-Trp^{6}]GnRH (▼), and [D-Ala^{6},*N*-Me-Leu^{7},Pro^{9}-NHEt]GnRH (□) in COS-1 cells transfected with [Gln^{301}]GnRHR, as described under "Materials and Methods."

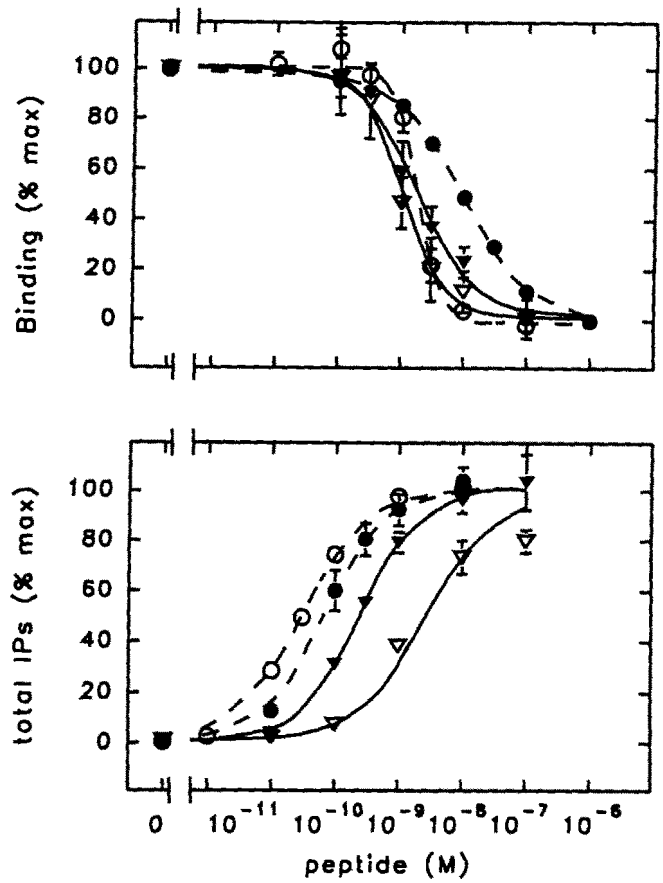

FIG. 6. **Two conformationally constrained analogues of GnRH exhibit high affinity for both wild type GnRHR and the [Gln^{301}]GnRHR mutant.** Binding of ^{125}I-[D-Ala^{6},*N*-Me-Leu^{7},Pro^{9}NHEt]-GnRH (*top panel*) in the presence of various concentrations of [D-Trp^{6},Pro^{9}-NHEt]GnRH (○, ▽) and [D-Trp^{6},Gln^{8},Pro^{9}-NHEt]GnRH (●, ▼) to membranes prepared from COS-1 cells transfected with wild type GnRHR (– – – –, ○, ●) or [Gln^{301}]GnRHR (—, ▽, ▼) was measured as described under "Materials and Methods." Data points are the mean ± S.E. of three to five experiments performed in triplicate. IP production (*lower panel*) in response to [D-Trp^{6},Pro^{9}-NHEt]GnRH (○, ▽) and [D-Trp^{6},Gln^{8},Pro^{9}-NHEt]GnRH (●, ▼) in COS-1 cells transfected with wild type GnRHR (– – – –, ○, ●) and [Gln^{301}]GnRHR (—, ▽, ▼) as described under "Materials and Methods." Data points are the means of two or three experiments performed in duplicate.

[Gln^{8}]GnRH in the wild type receptor is due only to the ability of Arg^{8} to stabilize the ligand conformation, then mutation of Glu^{301} to Gln must have disrupted the conformation of the ligand binding site such that it no longer recognizes the preferred ligand conformation. In this case, both constrained analogues should bind the wild type receptor with high affinity, and both constrained analogues should bind the mutant [Gln^{301}]GnRHR with low affinity, similar to that of the mutant receptor for unconstrained analogues. Our results indicate that the role of Arg^{8} is more complex than either a simple electrostatic interaction or simple stabilization of the ligand conformation. Both constrained peptides exhibited high apparent affinity for both the wild type receptor and the mutant [Gln^{301}]GnRHR. This shows that, although both Arg^{8} of the ligand and Glu^{301} of the receptor are necessary for high affinity binding of unconstrained ligands, preconstraining the conformation of the ligand allows high affinity binding in the absence of either or both of these charged residues. This necessity for Arg^{8} and Glu^{301} only in the absence of a conformational constraint suggests that an interaction which involves Arg^{8} and Glu^{301} induces the high affinity conformation of GnRH.

The ligand binding sites of heptahelical receptors for small ligands such as catecholamines are contained within the hydrophobic transmembrane helices (14, 15, 26). In contrast, large amino-terminal domains form the high affinity ligand binding sites of receptors for glycoprotein hormones (27). Peptide receptors, including the GnRHR, do not contain large amino-terminal domains and the ligand binding sites in these receptors appear to involve residues both in the transmembrane helices (28) and in the extracellular domains (29–31). We have shown that the Glu^{301} residue, located in the third extracellular loop of the GnRHR, plays a major role in determining ligand specificity. This confirms the importance of extracellular domains of receptors for peptide ligands.

The Glu^{301} residue is conserved in the rat and mouse GnRHRs, but in sheep and human GnRHRs it is replaced with Asp (2). This difference indicates that the length of the side chain is probably not critical for the interaction with GnRH, although it may contribute to some of the subtle differences in pharmacologies of mammalian GnRHRs (5). Preliminary experiments with mutants of the human GnRHR indicate that substitution of Asp^{302} with Glu does not affect interaction of the receptor with GnRH, whereas substitution with Asn affects the human GnRHR in much the same way as the Gln^{301} substitution affects the mouse GnRHR.

The [Gln^{301}]GnRHR shares some pharmacological characteristics with the chicken pituitary GnRHR in that GnRH and [Gln^{8}]GnRH display equal activity, whereas chicken GnRH II is more active (5). However, enhancement of activity resulting from D-amino acid substitutions at position 6 of GnRH is

greater in the [Gln^{301}]GnRHR (Table I) than in the chicken GnRHR (5), suggesting that the chicken GnRHR does not require the same ligand conformation as is required by mammalian GnRHRs. These results predict that although Glu^{301} is likely to be absent from the chicken GnRHR, the latter receptor may have other features which increase affinity to compensate for the absence of Arg^{8} in chicken GnRHs.

Our findings may apply to other receptors. Vasopressin receptors possess an acidic residue in their third extracellular loop (Glu^{299} in the human V2 receptor (32) and Glu^{323} in the rat V1 receptor (33)) which is not present at the corresponding position in the oxytocin receptor (34). Since vasopressins possess positively charged residues (Arg or Lys) which are replaced by neutral Leu in oxytocin, it is possible that a similar mechanism may determine the specificity of vasopressin and oxytocin receptors for their respective ligands.

In conclusion, we have identified a residue, Glu^{301}, in a mammalian GnRHR which confers specificity for the Arg at position 8 of mammalian GnRH. This specificity may result from an electrostatic interaction between the 2 residues. Interactions which involve the Glu^{301} residue of the receptor and the Arg^{8} residue of the ligand appear to induce changes in the conformation of the ligand.

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Glutamate 301 of the mouse gonadotropin-releasing hormone receptor confers specificity for arginine 8 of mammalian gonadotropin-releasing hormone.

C A Flanagan, I I Becker, J S Davidson, I K Wakefield, W Zhou, S C Sealfon and R P Millar

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