0026-895X/01/6006-1280–1287$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 60:1280–1287, 2001

Vol. 60, No. 6
882/944475
Printed in U.S.A.

Role of Aspartate7.32(302) of the Human Gonadotropin-Releasing Hormone Receptor in Stabilizing a High-Affinity Ligand Conformation

BERNHARD J. FROMME, ARIEH A. KATZ, ROGER W. ROESKE, ROBERT P. MILLAR, and COLLEEN A. FLANAGAN

Division of Medical Biochemistry (B.J.F., A.A.K., R.P.M., C.A.F.) and Department of Medicine (C.A.F.), University of Cape Town Faculty of Health Sciences, Observatory, South Africa; Indiana University School of Medicine, Indianapolis, Indiana (R.W.R.); and Medical Research Council Human Reproductive Sciences Unit, Edinburgh, Scotland, UK (R.P.M.)

Received February 2, 2001; accepted August 15, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Mammalian gonadotropin-releasing hormone (GnRH) receptors preferentially bind mammalian GnRH, which has Arg in position eight. The $Glu^{7.32(301)}$ residue, which determines selectivity of the mouse GnRH receptor for Arg^8-containing GnRH, is $Asp^{7.32(302)}$ in the human GnRH receptor. We have confirmed that $Asp^{7.32(302)}$ confers selectivity of the human GnRH receptor for Arg^8 of GnRH and investigated the mechanism of this specificity using site-directed mutagenesis and ligand modification. We find that although Arg^8 and $Asp^{7.32(302)}$ are required for high-affinity binding of GnRH, conformationally constrained peptides, with D-amino acid substitutions in position six or with a 6,7 γ-lactam, bind the human GnRH receptor with high affinity, which is independent of the presence of $Asp^{7.32(302)}$ in the receptor or Arg^8 in the ligand. The ability of the ligand constraints to compensate for the absence of both Arg^8 and $Asp^{7.32(302)}$ indicates that these residues both have roles in stabilizing a high affinity ligand conformation and that their roles are complementary. This suggests that the Arg^8 and $Asp^{7.32(302)}$ side chains interact to induce a high affinity conformation of native GnRH. Thus, $Asp^{7.32(302)}$ of the human GnRH receptor determines selectivity for mammalian GnRH by its ability to induce a high affinity conformation of its native ligand. However, this initial interaction seems not to contribute to the final ligand-receptor complex. We propose that Arg^8 interacts transiently with $Asp^{7.32(302)}$ to induce a high-affinity ligand conformation of GnRH, which then interacts with a binding pocket that is common for both constrained and unconstrained analogs of GnRH.

Gonadotropin-releasing hormone [GnRH, also called luteinizing hormone releasing hormone or luliberin] is a decapeptide that is synthesized in the hypothalamus and interacts with GnRH receptors on gonadotrope cells in the anterior pituitary. GnRH stimulates the biosynthesis and release of luteinizing hormone and follicle-stimulating hormone, which in turn are required for steroidogenesis and gametogenesis, respectively. Because of this central role in reproduction, GnRH analogs have been used in a variety of therapeutic applications (Millar et al., 1987).

Although an X-ray diffraction analysis of rhodopsin has recently been published (Palczewski et al., 2000), most other G protein-coupled receptors (GPCR) are more difficult to purify. Consequently, understanding of the structure of these GPCRs is likely to depend on indirect methods, such as computational modeling and mutagenesis, for some time to come. Considerable advances have been made in understanding how GnRH interacts with its receptor. In the human GnRH receptor, residues $Asp^{2.61(98)}$, $Trp^{2.64(101)}$, $Asn^{2.65(102)}$, $Lys^{3.32(121)}$, and $Asn^{5.61(212)}$ (residue numbering is described under *Materials and Methods*) have been shown to have roles in ligand binding (Zhou et al., 1995; Davidson et al., 1996; Flanagan et al., 2000; Hoffmann et al., 2000). Some of these receptor residues have been proposed to form part of the ligand binding pocket, interacting with the amino and carboxyl termini of GnRH in a computational model of the receptor-ligand complex (Sealfon et al., 1997). $Asp^{2.61(98)}$ is proposed to interact with His^2 of GnRH, whereas $Asn^{2.65(102)}$ interacts with Gly^{10}-NH_2. In the mouse GnRH receptor, $Glu^{7.32(301)}$ was shown to have a role in recognizing the Arg^8 residue of GnRH (Flanagan et al., 1994). However, $Glu^{7.32(301)}$ is not completely conserved in mammalian GnRH receptors. In the human and other nonrodent GnRH recep-

This research was supported by the Medical Research Council of South Africa, National Research Foundation of South Africa, University of Cape Town and the Wellcome Trust.

ABBREVIATIONS: GnRH, gonadotropin-releasing hormone; GPCR, G protein-coupled receptor; HPLC, high-performance liquid chromatography; PEI, polyethylenimine; IP, inositol phosphate; antagonist 26, [Ac-D-4-Cl-$Phe^{1,2}$,D-Trp^3,D-Lys^6,D-Ala^{10}-NH_2]-GnRH; antagonist 129–62, [Ac-D-3-(2-naphthyl) $alanine^1$,D-4-Cl-Phe^2,D-Trp^3,3-(3-pyridyl)$alanine^5$,6,7 γ-lactam, Ipr-Lys^8,D-Ala^{10}-NH_2]-GnRH; E_{max}, maximal agonist-stimulated inositol phosphate production; Ipr-Lys, N^{ϵ}-isopropyllysine; GnRH II, [His^5,Trp^7,Tyr^8]-GnRH.

tors the equivalent residue is Asp$^{7.32(302)}$ (Kakar et al., 1992; Chi et al., 1993; Illing et al., 1993; Sealfon et al., 1997; Cui et al., 2000). Although this is a conservative substitution, it is surprising that such a functionally important residue is not absolutely conserved. In the monoamine receptors, the Asp$^{3.32}$ residue, which is important for ligand binding, is conserved as Asp not only in different species but also in different receptor subtypes that recognize the same ligand and in different receptors that recognize distinct ligands ranging through acetylcholine, adrenaline, serotonin, and histamine (Probst et al., 1992).

In GnRH, Arg^{8} is required for high-affinity binding to mammalian GnRH receptors. Substitution of this residue decreases GnRH potency and affinity for the receptor (Millar et al., 1989). Mutation of the Glu$^{7.32(301)}$ residue of the mouse GnRH receptor to Gln decreased the receptor affinity for GnRH, but not for analogs with substitutions for Arg^{8} (Flanagan et al., 1994). Subsequent models of GnRH receptor-ligand complexes have incorporated an interaction of the acidic residue of the receptor with Arg^{8} of the ligand (Chauvin et al., 2000; Flanagan et al., 2000; Hoffmann et al., 2000). However, a GnRH analog with D-Trp substituted in position six showed only a small decrease in affinity for the Glu$^{7.32(301)}$Gln mouse receptor. This suggested that although Glu$^{7.32(301)}$ determines selectivity for native GnRH, the mechanism by which it does so may be more complex than a simple electrostatic interaction of Glu^{301} with Arg^{8}. It also indicates a need for caution in extrapolating experimental results to molecular models of GPCRs.

The lack of conservation indicates a need to determine whether Asp$^{7.32(302)}$ has the same function in the human GnRH receptor as Glu$^{7.32(301)}$ has in the mouse receptor. Furthermore, the incorporation of a direct interaction of Glu$^{7.32(301)}$/Asp$^{7.32(302)}$ with Arg^{8} in models of receptor-ligand complexes, despite evidence that a direct interaction may not always occur, shows that better definition of the mechanism by which the Asp$^{7.32(302)}$ determines binding specificity for GnRH is needed. We now show that mutating Asp$^{7.32(302)}$ in the human GnRH receptor decreases affinity for GnRH, but not for analogs with substitutions for Arg^{8}. In contrast, a series of peptides with different structural constraints that stabilize a high-affinity conformation of GnRH retain high affinity for the mutant receptor. This indicates that an electrostatic interaction is not involved in the binding of these constrained analogs. We also show that Arg^{8} is not required for high-affinity binding of constrained analogs. We interpret these results in terms of a sequential binding mechanism in which the Arg^{8} side chain of native GnRH interacts transiently with Asp$^{7.32(302)}$, before interacting with a final ligand binding pocket that also binds constrained analogs.

Materials and Methods

Consensus Residue Numbering Scheme. A consensus numbering scheme is used to facilitate the comparison of equivalent amino acid residues in the different rhodopsin-like GPCRs (Ballesteros and Weinstein, 1995). Amino acids were numbered relative to the most conserved residue in each transmembrane domain, which is assigned the number 50 (Fig. 1). Individual amino acid residues are identified by a generic identifier consisting of the transmembrane helix number, followed by the number representing its position relative to the most conserved residue in the helix. This is followed by its sequential number in the particular GPCR. For example, the most conserved residue in helix seven of the GnRH receptor is Pro, which is designated Pro$^{7.50}$. In the GnRH receptor, Pro$^{7.50}$ is residue number 320 and is designated Pro$^{7.50(320)}$. Asp^{302} is 18 amino acids closer to the amino-terminal than Pro$^{7.50}$ and is therefore designated Asp$^{7.32(302)}$.

Site Directed Mutagenesis. A polymerase chain reaction-based mutagenesis method was used to replace Asp$^{7.32(302)}$ with Asn in the human GnRH receptor. Primers contained the desired mutation and a silent restriction endonuclease site flanked by 12 bases of the wild-type receptor sequence on either side. Polymerase chain reaction products were digested with appropriate restriction enzymes, ligated using T4 DNA Ligase (Amersham Pharmacia Biotech, Piscataway, NJ), subcloned into the *Eco*RI and *Xho*I sites of the mammalian expression vector pcDNAI/AMP (Invitrogen, Carlsbad, CA), and transformed into competent XL-1 blue *Escherichia coli*. Plasmid DNA was extracted (Nucleobond Kit; Macherey-Nagel, Duren, Germany) from ampicillin-resistant clones and the mutation was confirmed by DNA sequencing (Epicentre Technologies, Madison, WI).

Transfection and Cell Culture. COS-1 cells were transiently transfected using the DEAE-Dextran method (Keown et al., 1990), as described previously (Millar et al., 1995). After transfection, COS-1 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum (Delta Bioproducts, Kempton Park, South Africa) and antibiotics (2 mg/ml streptomycin sulfate, 4000 U/ml sodium benzylpenicillin) in a 10% CO_2 incubator at 37°C.

GnRH Analogs. GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2), [His^{5},D-Tyr^{6}]-GnRH, [D-Ala^{6},*N*-Me-Leu^{7},Pro^{9}-NHEt]-GnRH, [D-Trp^{6},Pro^{9}-NHEt]-GnRH, [D-Trp^{6},Gln^{8},Pro^{9}-NHEt]-GnRH, [Gln^{8}]-GnRH, GnRH II ([His^{5},Trp^{7},Tyr^{8}]-GnRH), and antagonist 27 ([Ac-D-3-(2-naphthyl)alanine^{1},D-Me-4-Cl-Phe^{2},D-Trp^{3},Ipr-Lys^{5},D-Tyr^{6},D-Ala^{10}-NH_2]-GnRH) were prepared by conventional solid phase methodology and purified by preparative C-18 reverse phase high-performance liquid chromatography in our Cape Town laboratory. Antagonist 129–62([Ac-D-3-(2-naphthyl)alanine^{1},D-4-Cl-Phe^{2},D-Trp^{3},3-(3-pyridyl)alanine^{5},6,7 γ-lactam,Ipr-Lys^{8},D-Ala^{10}-NH_2]-GnRH) and [Glu^{8}]-GnRH were prepared by solid phase synthesis on a 4-methylbenzhydrylamine HCl resin using Boc/Benzyl chemistry. The Boc-γ-lactam (Freidinger et al., 1980) was added as one amino acid unit. After removal from the resin by hydrogen fluoride, the peptides were purified to homogeneity by reverse-phase high-performance liquid chromatography on a C-18 preparative column. Antagonist 26 ([Ac-D-4-Cl-Phe^{1,2},D-Trp^{3},D-Lys^{6},D-Ala^{10}-NH_2]-GnRH) was a gift from David Coy (Tulane University School of Medicine, New Orleans, LA). [6,7 γ-lactam]-GnRH was a gift from Roger Freidinger (Merck & Co., West Point, PA).

Phosphatidyl Inositol Hydrolysis. Transfected COS-1 cells (2×10^5 cells/well) in 12-well plates were incubated overnight with

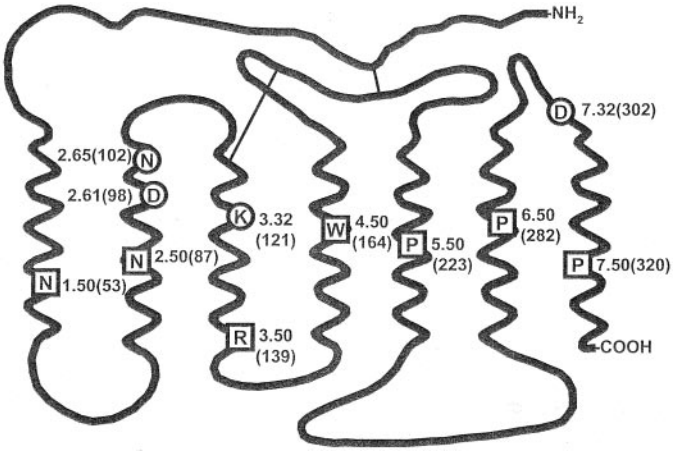

Fig. 1. Schematic diagram of the GnRH receptor. Circles indicate residues that are important for ligand binding. Squares indicate the most conserved residues in each transmembrane domain, which are reference residues in the consensus numbering scheme.

myo-[2-^{3}H]inositol (1 μCi/well; Amersham) in 0.5 ml Medium 199 (Invitrogen) with antibiotics. Labeled cells were incubated with various concentrations of ligand for 1 h at 37°C in the presence of LiCl as described previously (Millar et al., 1995). Aspirating the medium and addition of 10 mM formic acid (1 ml/well) terminated the incubation. Inositol phosphates were extracted from the formic acid extract on DOWEX-1 ion exchange columns and eluted into scintillation liquid (Quicksafe; Zinsser Analytical, Frankfurt, Germany) and the radioactivity was counted.

Radioligand Binding. Membrane binding assays were performed because this method makes it possible to optimize receptor concentration by varying the amount of transfected membranes used in the assay (Millar et al., 1995). The agonist peptide, [His^5,D-Tyr^6]-GnRH, was radioiodinated by the Chloramine-T method as described previously (Flanagan et al., 1998). Specific activity ranged between 900 and 1800 μCi/μg and 69% of the radioactivity could be bound by GnRH receptors. Using this high-affinity label allowed accurate determination of IC_{50} values for the mutant receptor, which had low total binding (Flanagan et al., 1998). Transfected COS-1 cells were homogenized in binding buffer (1 mM EDTA, 10 mM HEPES, pH 7.4, 0.1% bovine serum albumin) and centrifuged at 15,000*g* for 30 min at 4°C. The resultant crude membrane pellet was resuspended in binding buffer. The membrane suspension was incubated overnight at 4°C with ^{125}I-[His^5,D-Tyr^6]-GnRH (50,000 cpm, 50 pM) and varying concentrations of unlabeled GnRH analogs. We have found previously that equilibrium binding is achieved after 21 h and stable for up to 30 h (Flanagan et al., 1998). The incubation was terminated by the addition of cold polyethylenimine (0.01%; PEI) and immediate filtration through glass-fiber filters (GF/C; Whatman, Maidstone, UK) which were presoaked in 1% PEI. The filters were washed twice with 0.01% PEI and the retained radioactivity was counted. Nonspecific binding was determined in the presence of 1 μM antagonist 27.

Data Reduction. IP assays were performed at least three times in duplicate and competition binding assays in triplicate. Four-parameter nonlinear curve fitting (Prism; GraphPad Software Inc., San Diego, CA) was used to estimate the peptide concentrations required to stimulate half-maximal IP production (EC_{50}) and to half-maximally inhibit the binding of the radioligand (IC_{50}). K_i values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). K_d and B_{max} values were determined using nonlinear curve-fitting (Prism) of homologous competition binding assays (Munson and Rodbard, 1980; Klotz, 1982; Motulsky, 1999). The high nonspecific binding of the ^{125}I-[His^5,D-Tyr^6]-GnRH tracer at high concentrations makes saturation binding assays unreliable. Data in figures are from single experiments that are representative of at least three independent experiments. Data in tables are the mean ± S.E. of at least three experiments. *P* values were calculated using unpaired two-tailed *t* tests performed on the negative log of K_i and EC_{50} values, and paired two-tailed *t* tests on the E_{max} counts.

Results

Mutation of Asp$^{7.32(302)}$ Decreases Affinity for GnRH. The wild-type GnRH receptor bound [His^5,D-Tyr^6]-GnRH, which was used as a radiolabeled ligand, with high affinity (K_d = 0.35 ± 0.06 nM). The Asp$^{7.32(302)}$Asn mutant receptor showed 2.8-fold lower affinity for [His^5,D-Tyr^6]-GnRH (K_d 0.99 nM ± 0.01 nM). Receptor number was unaffected by the Asp$^{7.32(302)}$ mutation (wild-type, 1.31 ± 0.23 × 10^5 sites/cell; Asp$^{7.32(302)}$Asn mutant, 1.31 ± 0.06 × 10^5 sites/cell). The similar expression suggests that the lower total binding of the mutant receptor reported in initial experiments (Flanagan et al., 1998) results from the slightly decreased affinity for ^{125}I-[His^5,D-Tyr^6]-GnRH.

In competitive ligand binding assays, the affinities of the wild-type human GnRH receptor for uncharged, [Gln^8]-GnRH (K_i = 923 ± 222 nM) and negatively charged, [Glu^8]-GnRH (>10,000 nM) were lower than that for Arg^8-containing GnRH (K_i = 6.79 ± 1.08 nM) (Table 1, Fig. 2). This shows that the human GnRH receptor preferentially binds Arg^8-containing GnRH. Mutating Asp$^{7.32(302)}$ to Asn decreased affinity for native GnRH (31.2-fold), but the affinities for uncharged [Gln^8]-GnRH or negatively charged [Glu^8]-GnRH were unchanged (Table 1, Fig. 2). This indicates that Asp$^{7.32(302)}$ determines the specificity of the wild-type GnRH receptor for native Arg^8-containing GnRH. This is consistent with the proposal that the carboxyl side chain of Asp$^{7.32(302)}$ may be involved in an electrostatic interaction with the positively charged Arg^8 side chain in GnRH. However, the mutant receptor retained higher affinity for native GnRH than for [Gln^8]-GnRH (6-fold) and did not show enhanced affinity for [Glu^8]-GnRH. This result suggests that the mechanism by which the receptor selects for Arg^8-containing GnRH may be more complex than a simple electrostatic interaction of Arg^8 with Asp$^{7.32(302)}$.

GnRH Analogs with D-Amino Acid Substitutions in Position Six of GnRH Enhance Affinity and Overcome

TABLE 1

Summary of ligand binding results

Competition binding assays were performed on COS-1 cells transiently transfected with wild type or mutant GnRH receptors, using ^{125}I-[His^5, D-Tyr^6]-GnRH and various concentrations of unlabeled ligands as described under *Materials and Methods*. K_i values are presented as mean ± S.E. Statistical analyses were performed using pK_i values as described under *Materials and Methods*. The fold change was calculated as the ratio of the K_i values of the mutant and wild-type receptors.

GnRH analog	K_i		Fold change
	Wild-Type	Mutant	
	nM		
GnRH[a]	6.79 ± 1.08	212 ± 40.6**	31.2
[Gln^8]-GnRH	923 ± 222	1240 ± 227	1.35
[Glu^8]-GnRH	>10 000	>10 000	—
[His^5,D-Tyr^6]-GnRH	0.48 ± 0.12[b]	1.36 ± 0.20*[b]	2.83
[D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]-GnRH	0.97 ± 0.35	1.55 ± 0.09	1.60
[D-Trp^6,Pro^9-NHEt]-GnRH	0.15 ± 0.04	0.27 ± 0.06	1.80
[D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH	1.86 ± 0.19	1.67 ± 0.43	0.82
[6,7 γ-lactam]-GnRH	1.37 ± 0.07	4.66 ± 0.36**	3.40
GnRH II ([His^5,Trp^7,Tyr^8]-GnRH)	193.7 ± 28.5	213.3 ± 72.4	1.10
Antagonist 129-62	0.86 ± 0.21	1.52 ± 0.28	1.76
Antagonist 26	0.80 ± 0.13	1.13 ± 0.19	1.41

*, Significantly different from wild type, $p < 0.05$.
**, Significantly different from wild type, $p < 0.001$.
[a] GnRH sequence: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2.
[b] K_d values are reported for the homologous ligand, [His^5,D-Tyr^6]-GnRH.

the Absence of Arg^8 and/or Asp$^{7.32(302)}$. We found previously that binding of the conformationally constrained analog, [D-Trp^6,Pro^9-NHEt]-GnRH, was not affected by the Glu$^{7.32(301)}$ Gln mutation in the mouse GnRH receptor (Flanagan et al., 1994). To test whether this is a general phenomenon in mammalian GnRH receptors, binding affinities of a series of GnRH analogs with different D-amino acid substitutions in position six were characterized in the wild-type human receptor and in the Asp$^{7.32(302)}$Asn mutant (Fig. 3). As expected for the wild-type receptor (Karten and Rivier, 1986; Sealfon et al., 1997), three GnRH analogs, [His^5,D-Tyr^6]-GnRH, [D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]-GnRH, and [D-Trp^6,Pro^9-NHEt]-GnRH, showed higher affinity (7.0- to 45-fold) than native GnRH (Tables 1 and 2, Fig. 3). The affinity of the wild-type receptor for the uncharged but conformationally constrained analog [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH (K_i = 1.9 ± 0.19 nM) was 486-fold higher than the affinity for [Gln^8]-GnRH (K_i = 923 ± 222 nM) (Table 1). This shows that, in the wild-type receptor, incorporation of a D-amino acid in position six enhances the affinity of [Gln^8]-GnRH more than the affinity of Arg^8 -containing GnRH and thus compensates for the absence of Arg^8 (Table 2). However, the affinity of [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH (K_i = 1.9 ± 0.19 nM) remained 13-fold lower than the affinity of [D-Trp^6,Pro^9-NHEt]-GnRH (K_i = 0.15 ± 0.04 nM).

All four GnRH analogs with D-amino acid substitutions in position six had similar high affinity for the Asp$^{7.32(302)}$Asn mutant receptor compared with the wild-type receptor (Table 1). In the mutant receptor, the affinities of the three analogs with D-amino acids in position six and Arg in position eight were 137- to 784-fold higher than the affinity for (native) GnRH (Table 2). The affinity of the uncharged [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH was 744-fold higher than for [Gln^8]-GnRH (Fig. 3, Table 2).

Incorporation of a 6,7 γ-Lactam Enhances Binding to the Receptor. Because D-amino acid substitutions in position six are thought to stabilize a high-affinity conformation of GnRH (Monahan et al., 1973), the high affinity of the mutant receptor for peptides with D-amino acids in position six suggests that conformationally constrained peptides may be less sensitive to the Asp$^{7.32(302)}$ mutation. However, part of the enhanced affinity may be contributed by an interaction of the amino acid side chain (e.g., D-Trp) with a receptor residue. To test whether the high affinity is caused predominantly by the conformational constraint of the D-amino acid, peptides with a conformational constraint, in which there is no side chain, were examined. Introduction of a γ-lactam moiety in place of residues six and seven is reported to impose a peptide conformation, which is similar to that stabilized by D-amino acid modifications (Freidinger et al., 1980).

Consistent with the previous report (Freidinger et al., 1980), [6,7 γ-lactam]-GnRH exhibited higher affinity than GnRH for the wild-type GnRH receptor (5.0-fold; Table 2). This result is similar to the increase found with D-amino acid substitutions in position six of GnRH (Table 2). [6,7 γ-Lactam]-GnRH also had high affinity for the Asp$^{7.32(302)}$Asn mutant GnRH receptor (K_i = 4.66 ± 0.36 nM; Table 1), which was 45.5-fold higher than the affinity for native GnRH (Table 2). This shows that the γ-lactam constraint enhances the affinity of GnRH for both the wild-type and mutant receptors (Fig. 4, Table 1). [6,7 γ-Lactam]-GnRH had similar affinity for both the wild-type receptor (K_i = 1.37 ± 0.07 nM) and the mutant receptor (K_i = 4.66 ± 0.36 nM, Table 1). Similar to the D-amino acid substitution in position six, incorporation of 6,7 γ-lactam enhanced binding affinity more in the mutant receptor than in the wild-type receptor. These results show that when the conformation of GnRH is constrained (6,7 γ-lactam or D-amino acid in position six), Asp$^{7.32(302)}$ of the

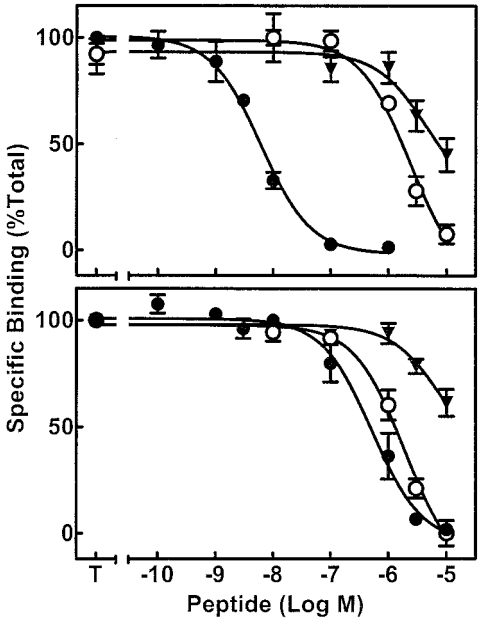

Fig. 2. Competition binding of GnRH and GnRH analogs in human wild-type and Asp$^{7.32(302)}$Asn mutant GnRH receptors. COS-1 cell membranes expressing the human wild-type (top) and Asp$^{7.32(302)}$Asn mutant (bottom) GnRH receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH in the presence of various concentrations of GnRH (●), [Gln^8]-GnRH (○), and [Glu^8]-GnRH (▼). Data are presented as mean ± S.E. of representative experiments performed in triplicate and expressed as percentage of specific binding in the absence of unlabeled ligand (T).

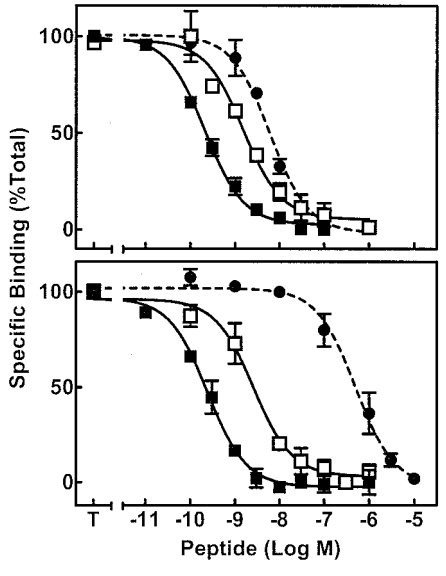

Fig. 3. Competition binding of GnRH analogs with D-amino acid substitutions in position six. COS-1 cell membranes expressing the human wild-type (top) and Asp$^{7.32(302)}$Asn mutant (bottom) GnRH receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH in the presence of various concentrations of GnRH (●), [D-Trp^6,Pro^9-NHEt]-GnRH (■), and [D-Trp^6, Gln^8,Pro^9-NHEt]-GnRH (□). Data are presented as mean ± S.E. of representative experiments performed in triplicate and expressed as percentage of specific binding in the absence of unlabeled ligand (T).

receptor is not required for high-affinity binding. Furthermore, the high affinity of [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH, which has a constraint but no Arg^8, shows that Arg^8 also is not required for high-affinity binding of conformationally constrained agonists.

Another class of conformationally constrained GnRH analogs includes the GnRH antagonists. The novel antagonist, antagonist 129–62, which has a 6,7 γ-lactam, and antagonist 26, which has D-Lys^6, had similar high affinities for the wild-type and mutant receptors (Table 1, Fig. 5).

GnRH II, which occurs naturally in the human brain (White et al., 1998) does not contain Arg^8, but binds the GnRH receptor with higher affinity (K_i = 193.7 ± 28.5 nM) than [Gln^8]-GnRH (K_i = 923 ± 222 nM; Table 1, Fig. 4) or [Tyr^8]-GnRH (Millar et al., 1989). This suggests that the combination of substitutions in positions five, seven, and eight allows the peptide to bind with relatively high affinity, independently of interactions that involve Arg^8, possibly by stabilizing a high affinity conformation (de L Milton et al., 1983). GnRH II had similar affinity for the wild-type (K_i = 193.7 ± 28.5 nM) and mutant receptors (K_i = 213.3 ± 72.4 nM; Table 1). Consequently, the Asp$^{7.32(302)}$Asn mutant receptor retained higher affinity for GnRH II (K_i = 213.3 ± 72.4 nM) than for [Gln^8]-GnRH (K_i = 1240 ± 227 nM).

In summary, these results show that mutating Asp$^{7.32(302)}$ of the human GnRH receptor to Asn decreases the affinity for GnRH in a manner that is specific to an interaction with Arg^8, and that a specific conformation of GnRH is important for high-affinity binding of GnRH to its receptor.

Decreased IP Production in the Asp$^{7.32(302)}$Asn Mutant GnRH receptor. The Asp$^{7.32(302)}$Asn mutant GnRH receptor coupled to the IP signaling pathway (Fig. 6). GnRH displayed a 44-fold decrease in potency at the mutant receptor (EC_{50} = 12.6 ± 1.78 nM) relative to the wild-type receptor (EC_{50} = 0.29 ± 0.07 nM; Fig. 6). This decrease in potency is consistent with the decreased affinity of the mutant receptor for native GnRH. However, the mutant receptor also exhibited a decreased E_{max} value for GnRH (Fig. 6, Table 3), suggesting that the mutation may induce partial uncoupling of the receptor from intracellular signaling. Surprisingly, ligands that showed no decrease in affinity for the mutant receptor also exhibited decreased IP production in the mutant receptor (Table 3, Fig. 6). This shows that the effect of the mutation on cytosolic signaling is distinct from its effect on binding affinity for GnRH.

Discussion

The basic Arg residue in position eight of GnRH is required for high-affinity binding to mammalian GnRH receptors (Millar et al., 1989). The proposal that Arg^8 may be involved in an electrostatic interaction with an acidic residue in the GnRH receptor (Hazum, 1987) was examined in the mouse GnRH receptor, where it was found that the Glu$^{7.32(301)}$ residue confers specificity for GnRH with Arg in position eight (Flanagan et al., 1994). Despite the demonstrated functional importance of Glu$^{7.32(301)}$, this residue is not conserved in the human GnRH receptor, which has Asp$^{7.32(302)}$ instead (Chi et al., 1993). The carboxyl side chain of both residues suggests that Asp$^{7.32(302)}$ can potentially perform the same functions in the human GnRH receptor as Glu$^{7.32(301)}$ does in the mouse receptor and computational models of both rodent and human receptors have

TABLE 2

Enhancement of GnRH affinity by conformational constraints

The fold enhancement of peptide affinity by conformational constraints was calculated as the ratio of the K_i value of the parent peptide (GnRH or [Gln^8]-GnRH) to the K_i value of the constrained peptide in the human (wild-type) receptor or Asp$^{7.32(302)}$Asn mutant GnRH receptor.

Peptide	Wild-Type		Mutant	
	K_i	Fold enhancement	K_i	Fold enhancement
	nM		*nM*	
GnRH	6.8[a]		212	
[D-Ala^6,N-Me-Leu^7,Pro^9-NHEt]-GnRH	0.97*	7.0	1.55*	137
[His^5,D-Tyr^6]-GnRH	0.48*	14.2	1.36*	156
[D-Trp^6,Pro^9-NHEt]-GnRH	0.15*	45.3	0.27*	784
[6,7 γ-lactam]-GnRH	1.37*	5.0	4.66*	45.5
[Gln^8]-GnRH	923		1240	
[D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH	1.86**	485.5	1.67**	744

[a] K_i values are the same as presented in Table 1.
*, Significantly different from GnRH, $p < 0.01$.
**, Significantly different from [Gln^8]-GnRH, $p < 0.001$.

TABLE 3

Summary of GnRH receptor agonist-stimulated IP accumulation

EC_{50} and E_{max} values are presented as mean ± S.E. Statistical analyses were performed as described under *Materials and Methods.* The fold change was calculated as the ratio of the EC_{50} values in the mutant and wild-type receptors. E_{max} in the mutant is expressed as percentage of E_{max} stimulated by the same ligand in wild-type receptor in the same experiment.

GnRH analog	EC_{50}		Fold change	E_{max}
	Wild-Type	Mutant		
	nM			*% wt*
GnRH	0.29 ± 0.07	12.6 ± 1.80**	43.5	68.7 ± 8.32*
[Gln^8]-GnRH	5.19 ± 1.13	17.2 ± 2.84*	3.3	69.3 ± 4.20*
GnRH II	1.75 ± 0.55	6.66 ± 1.62*	3.8	71.3 ± 7.70*
[D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]-GnRH	0.06 ± 0.002	0.31 ± 0.17	5.2	76.0 ± 2.89

*, Significantly different from wild-type receptor, $p < 0.05$.
**, Significantly different from wild-type receptor, $p < 0.001$.

incorporated interactions of Glu or Asp with Arg^8. However, the study in the mouse receptor suggested that the mechanism of receptor selectivity for Arg^8-containing GnRH may be more complex than a simple electrostatic interaction in the receptor ligand complex. This demonstrates a need for detailed investigation of the mechanism by which Asp$^{7.32(302)}$ determines specificity for Arg^8. We show that an electrostatic interaction is not required for high-affinity binding of certain GnRH analogs and we propose that Asp$^{7.32(302)}$ may stabilize a high-affinity conformation of GnRH.

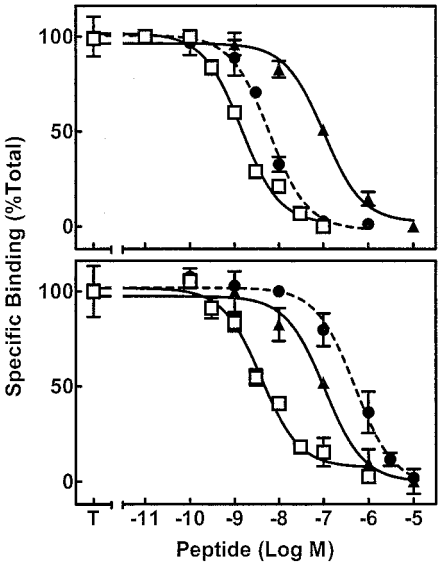

Fig. 4. Competition binding of GnRH analogs with and without 6,7 γ-lactam conformational constraint. COS-1 cell membranes expressing the human wild-type (top) and Asp$^{7.32(302)}$Asn mutant (bottom) GnRH receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH in the presence of various concentrations of GnRH (●), [6,7 γ-lactam]-GnRH (□), and GnRH II (▲). Data are presented as mean ± S.E. of representative experiments performed in triplicate and expressed as percentage specific binding in the absence of unlabeled ligand (T).

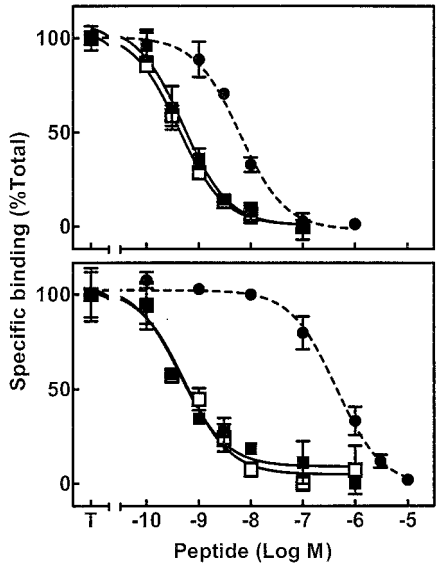

Fig. 5. Competition binding of GnRH antagonists. COS-1 cell membranes expressing the human wild-type (top) and Asp$^{7.32(302)}$Asn mutant (bottom) GnRH receptors were incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH in the presence of various concentrations of GnRH (●), antagonist 26 (■), and antagonist 129–62 (□). Data are presented as mean ± S.E. of representative experiments performed in triplicate and expressed as percentage specific binding in the absence of unlabeled ligand (T).

Asp$^{7.32(302)}$ of the Human GnRH Receptor Determines Selectivity for GnRH. The decreased affinity of the Asp$^{7.32(302)}$Asn mutant for native GnRH, but not for [Gln^8]-GnRH shows a loss of specificity for Arg^8 of GnRH. This shows that, like the Glu$^{7.32(301)}$ residue of the mouse GnRH receptor, the Asp$^{7.32(302)}$ side chain determines receptor preference for the Arg^8 side chain of GnRH. However, the 31-fold decrease in the affinity of the Asp$^{7.32(302)}$Asn mutant for GnRH is smaller than would be expected for a loss of an electrostatic interaction (Wells et al., 1987). The low affinity of the negatively charged ligand, [Glu^8]-GnRH could potentially result from a repulsive interaction with the negatively charged Asp$^{7.32(302)}$ of the wild-type receptor, but the Asp$^{7.32(302)}$Asn human GnRH receptor mutant did not show increased affinity for [Glu^8]-GnRH. This suggests that the mutation does not remove an unfavourable interaction with [Glu^8]-GnRH; consequently, high-affinity binding of native GnRH may not arise from an electrostatic interaction of the Arg^8 and Asp$^{7.32(302)}$ side chains.

Conformational Constraints Enhance GnRH Binding to the Wild-Type GnRH Receptor. Arg^8 is proposed to stabilize an active conformation of GnRH (Shinitzky et al., 1976), which consists of a β-II-bend involving the Tyr^5-Gly^6-Leu^7-Arg^8 residues (Monahan et al., 1973). Incorporation of a D-amino acid in position six (Momany, 1976) or a γ-lactam in positions six and seven (Freidinger et al., 1980) is proposed to stabilize this conformation and results in an increased GnRH potency (Monahan et al., 1973; Freidinger et al., 1980). The current study has shown that GnRH analogs with D-amino acid substitutions in position six, or with a 6,7 γ-lactam, have higher affinities than native GnRH for the wild-type human GnRH receptor. This shows that the human GnRH receptor has enhanced affinity for the conformational state of GnRH that is stabilized by incorporating either constraint.

To test whether preferential binding of Arg^8-containing GnRH is retained in the presence of a conformational constraint in the wild-type receptor, affinities of conformationally constrained peptides with Arg^8 or Gln^8 ([D-Trp^6,Pro^9-NHEt]-GnRH and [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH) were compared. The modification enhanced the affinity of [Gln^8]-

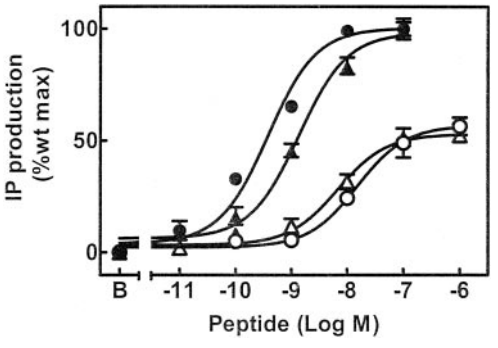

Fig. 6. GnRH-stimulated IP production. COS-1 cells transiently transfected with the wild-type human GnRH receptor (filled symbols) and with the Asp$^{7.32(302)}$Asn mutant (open symbols) were incubated with various concentrations of GnRH (●, ○) and GnRH II (▲, △). Data are presented as mean ± S.E. of a representative experiment performed in duplicate and expressed as percentage of E_{max} stimulated by each peptide in the wild-type receptor. EC_{50} values in this experiment were 0.38 nM for GnRH in the wild-type, 15.1 nM for GnRH in the mutant, 1.36 nM for GnRH II in the wild-type, and 6.76 nM for GnRH II in the mutant.

GnRH more than that of GnRH (Table 2). Thus, a conformational constraint not only enhances the affinity of [Gln^8]-GnRH but also compensates for the absence of Arg^8. This shows that Arg^8 is not required for high-affinity binding of conformationally constrained analogs to the wild-type human GnRH receptor.

Constrained Ligands Retain High Affinity for the $Asp^{7.32(302)}$Asn Mutant. In contrast to native GnRH, GnRH analogs with conformational constraints retained high affinity for the $Asp^{7.32(302)}$Asn mutant receptor. Because of the lower affinity of the mutant receptor for GnRH, the enhancement of the affinity of the conformationally constrained analogs, compared with GnRH, was greater in the mutant receptor than in the wild-type receptor. The preservation of high affinity for constrained peptides in the mutant receptor shows that the $Asp^{7.32(302)}$ side chain is not required for high-affinity binding of conformationally constrained GnRH analogs.

Ligand Constraint Compensates for the Absence of Both Arg^8 and $Asp^{7.32(302)}$. The high-affinity binding of the Gln^8-containing analog, [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH, to the mutant receptor shows that the conformational constraint can compensate for the simultaneous absence of both Arg^8 in the ligand and $Asp^{7.32(302)}$ in the receptor. This result suggests that native GnRH interacts with the receptor differently than conformationally constrained GnRH analogs. Thus, native GnRH and conformationally constrained GnRH analogs may occupy different (although overlapping) binding pockets on the receptor. Two other GnRH receptor residues, $Asp^{2.61(98)}$ and $Asn^{2.65(102)}$, determine recognition of His^2 and Gly-NH_2 of GnRH, respectively (Davidson et al., 1996a; Flanagan et al., 2000). Comprehensive analysis shows that the interaction of these receptor residues with native GnRH is similar to their interaction with constrained analogs. Mutation of these residues decreases receptor recognition of native and constrained analogs of GnRH to the same extent, suggesting that both native GnRH and constrained analogs interact with these residues (Davidson et al., 1996; Flanagan et al., 2000). Thus, the ability of the ligand conformational constraint to overcome a receptor mutation is specific for the $Asp^{7.32(302)}$Asn mutant.

$Asp^{7.32(302)}$ and Arg^8 Induce a High-Affinity Conformation of GnRH. We have shown that substituting Arg^8 of native GnRH or $Asp^{7.32(302)}$ of the receptor decreases binding affinity. The lack of an additive effect when both substitutions are combined suggests that these side chains interact with each other. However, conformational constraint of the ligand reverses the loss of affinity due to substitution of Arg^8 and/or $Asp^{7.32(302)}$. This suggests that both residues have roles in stabilizing a high affinity conformation of unconstrained GnRH. Arg^8 has been proposed to have two distinct roles in high-affinity binding: an intramolecular interaction that stabilizes a high-affinity peptide conformation (Shinitzky et al., 1976) and an intermolecular electrostatic interaction with an acidic group in the receptor (Hazum, 1987; Flanagan et al., 1994). The current results suggest that Arg^8 both stabilizes peptide conformation and interacts with $Asp^{7.32(302)}$, and that $Asp^{7.32(302)}$ also affects peptide conformation. This, in turn, suggests that an interaction of Arg^8 with $Asp^{7.32(302)}$ affects peptide conformation. The similar affinities of constrained peptides for the wild-type and mutant receptors (with and without $Asp^{7.32(302)}$) suggest that once the ligand is in a high-affinity conformation, the putative Arg^8-$Asp^{7.32(302)}$ interaction does not contribute to the binding energy of the final ligand-receptor complex. Although our results suggest that an interaction between Arg^8 and $Asp^{7.32(302)}$ may be required to induce a high-affinity conformation in unconstrained, native GnRH, the absence of this interaction with constrained analogs suggests that the interaction that induces the high-affinity conformation is transient. It has been suggested that residues on the extracellular surface of the TRH receptor form a initial ligand recognition site (Perlman et al., 1997) and that TRH binds sequentially with the surface binding site, and then with the transmembrane binding pocket (Colson et al., 1998). GnRH may interact initially with $Asp^{7.32(302)}$ and then move to a final binding pocket, which involves $Asn^{2.65(102)}$ and $Asp^{2.61(98)}$ (Davidson et al., 1996; Flanagan et al., 2000), after assuming a high affinity conformation. Thus, contrary to the initial hypothesis of an electrostatic interaction in the ligand-receptor complex, we show that the basis of receptor selectivity for mammalian GnRH seems to be the ability of $Asp^{7.32(302)}$ to induce a high affinity conformation in native GnRH.

Cytosolic signaling assays showed that GnRH had decreased potency at the $Asp^{7.32(302)}$Asn mutant receptor, consistent with its decreased affinity. However, the E_{max} value for GnRH was lower in the mutant receptor, suggesting that the mutation destabilizes the activated receptor conformation (Samama et al., 1993). Several agonists, which showed no decrease in affinity for the mutant receptor, nevertheless showed decreased stimulation of IP production. This suggests that the $Asp^{7.32(302)}$ side chain has a role stabilizing the activated receptor conformation. The apparent decreased efficacy of ligands that had unchanged affinity suggests that this function is distinct from its role in ligand selectivity. A previous study reported that mutagenesis of extracellular loop three of the β_2-adrenergic receptor also affected receptor activation (Zhao et al., 1998).

In conclusion, the wild-type human GnRH receptor recognizes and binds ligands in a specific conformation that can be stabilized by ligand modifications. We show that the $Asp^{7.32(302)}$ side chain determines selectivity for Arg^8-containing GnRH. However, certain ligand conformational constraints overcome the decrease in affinity that results from substitution of Arg^8 and $Asp^{7.32(302)}$. This suggests that $Asp^{7.32(302)}$ determines selectivity for Arg^8-containing GnRH by its ability to induce a high-affinity conformation in the ligand. We propose that unconstrained, Arg^8-containing, native GnRH interacts transiently with $Asp^{7.32(302)}$, which induces a high affinity conformation in the ligand, before it interacts with a final ligand binding pocket, which excludes $Asp^{7.32(302)}$. This further definition of the mechanism of ligand recognition improves our conceptual models of GnRH receptor-ligand interaction.

Acknowledgments

We thank David Coy for antagonist 26 and Roger Freidinger for [6,7 γ-lactam]-GnRH and Boc-γ lactam.

References

Ballesteros JA and Weinstein H (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors, in *Methods in Neurosciences*, *Receptor Molecular Biology* (Sealfon SC ed) pp 366–428, Academic Press, San Diego.

Chauvin S, Berault A, Lerrant Y, Hibert M, and Counis R (2000) Functional importance of transmembrane helix 6 Trp(279) and exoloop 3 Val(299) of rat gonadotropin-releasing hormone receptor. *Mol Pharmacol* **57:**625–633.
Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.
Chi L, Zhou W, Prikhozhan A, Flanagan C, Davidson JS, Golembo M, Illing N, Millar RP, and Sealfon SC (1993) Cloning and characterization of the human GnRH receptor. *Mol Cell Endocrinol* **91:**R1–R6.
Colson AO, Perlman JH, Smolyar A, Gershengorn MC, and Osman R (1998) Static and dynamic roles of extracellular loops in G-protein-coupled receptors: a mechanism for sequential binding of thyrotropin-releasing hormone to its receptor. *Biophys J* **74:**1087–1100.
Cui J, Smith RG, Mount GR, Lo JL, Yu J, Walsh TF, Singh SB, DeVita RJ, Goulet MT, Schaeffer JM, and Cheng K (2000) Identification of Phe313 of the gonadotropin-releasing hormone (GnRH) receptor as a site critical for the binding of nonpeptide GnRH antagonists. *Mol Endocrinol* **14:**671–681.
Davidson JS, McArdle CA, Davies P, Elario R, Flanagan CA, and Millar RP (1996) Asn102 of the gonadotropin-releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycinamide. *J Biol Chem* **271:**15510–15514.
de L Milton RC, King JA, Badminton MN, Tobler CJ, Lindsey GG, Fridkin M and Millar RP (1983) Comparative structure-activity studies on mammalian [Arg8] LH-RH and chicken [Gln8] LH-RH by fluorimetric titration. *Biochem Biophys Res Commun* **111:**1082–1088.
Flanagan CA, Becker II, Davidson JS, Wakefield IK, Zhou W, Sealfon SC, and Millar RP (1994) Glutamate 301 of the mouse gonadotropin-releasing hormone receptor confers specificity for arginine 8 of mammalian gonadotropin-releasing hormone. *J Biol Chem* **269:**22636–22641.
Flanagan CA, Fromme BJ, Davidson JS, and Millar RP (1998) A high affinity gonadotropin-releasing hormone (GnRH) tracer, radioiodinated at position 6, facilitates analysis of mutant GnRH receptors. *Endocrinology* **139:**4115–4119.
Flanagan CA, Rodic V, Konvicka K, Yuen T, Chi L, Rivier JE, Millar RP, Weinstein H, and Sealfon SC (2000) Multiple interactions of the Asp(2.61(98)) side chain of the gonadotropin-releasing hormone receptor contribute differentially to ligand interaction. *Biochemistry* **39:**8133–8141.
Freidinger RM, Veber DF, Perlow DS, Brooks JR, and Saperstein R (1980) Bioactive conformation of luteinizing hormone-releasing hormone: evidence from a conformationally constrained analog. *Science (Wash DC)* **210:**656–658.
Hazum E (1987) Binding properties of solubilized gonadotropin-releasing hormone receptor: role of carboxylic groups. *Biochemistry* **26:**7011–7014.
Hoffmann SH, ter Laak T, Kuhne R, Reilander H and Beckers T (2000) Residues within transmembrane helices 2 and 5 of the human gonadotropin-releasing hormone receptor contribute to agonist and antagonist binding. *Mol Endocrinol* **14:**1099–1115.
Illing N, Jacobs GF, Becker II, Flanagan CA, Davidson JS, Eales A, Zhou W, Sealfon SC, and Millar RP (1993) Comparative sequence analysis and functional characterization of the cloned sheep gonadotropin-releasing hormone receptor reveal differences in primary structure and ligand specificity among mammalian receptors. *Biochem Biophys Res Commun* **196:**745–751.
Kakar SS, Musgrove LC, Devor DC, Sellers JC, and Neill JD (1992) Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor. *Biochem Biophys Res Commun* **189:**289–295.
Karten MJ and Rivier JE (1986) Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: rationale and perspective. *Endocr Rev* **7:**44–66.
Keown WA, Campbell CR, and Kucherlapati RS (1990) Methods for introducing DNA into mammalian cells. *Methods Enzymol* **185:**527–537.
Klotz IM (1982) Numbers of receptor sites from Scatchard graphs: facts and fantasies. *Science (Wash DC)* **217:**1247–1249.
Millar RP, Davidson J, Flanagan CA, and Wakefield I (1995) Ligand binding and second messenger assays for cloned Gq/G11-coupled neuropeptide receptors: the GnRH receptor, in *Methods in Neurosciences*, *Receptor Molecular Biology* (Sealfon SC ed) pp 145–162, Academic Press, San Diego.
Millar RP, Flanagan CA, Milton RC and King JA (1989) Chimeric analogues of vertebrate gonadotropin-releasing hormones comprising substitutions of the variant amino acids in positions 5, 7, and 8. Characterization of requirements for receptor binding and gonadotropin release in mammalian and avian pituitary gonadotropes. *J Biol Chem* **264:**21007–21013.
Millar RP, King JA, Davidson JS, and Milton RC (1987) Gonadotrophin-releasing hormone–diversity of functions and clinical applications. *S Afr Med J* **72:**748–755.
Momany FA (1976) Conformational energy analysis of the molecule, luteinizing hormone- releasing hormone. I. Native decapeptide. *J Am Chem Soc* **98:**2990–2996.
Monahan MW, Amoss MS, Anderson HA, and Vale W (1973) Synthetic analogs of the hypothalamic luteinizing hormone releasing factor with increased agonist or antagonist properties. *Biochemistry* **12:**4616–4620.
Motulsky H (1999) *Analyzing Data with GraphPad Prism*. GraphPad Software Inc., San Diego.
Munson PJ and Rodbard D (1980) Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107:**220–239.
Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, et al. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science (Wash DC)* **289:**739–745.
Perlman JH, Colson AO, Jain R, Czyzewski B, Cohen LA, Osman R, and Gershengorn MC (1997) Role of the extracellular loops of the thyrotropin-releasing hormone receptor: evidence for an initial interaction with thyrotropin-releasing hormone. *Biochemistry* **36:**15670–15676.
Probst WC, Snyder LA, Schuster DI, Brosius J, and Sealfon SC (1992) Sequence alignment of the G-protein coupled receptor superfamily. *DNA Cell Biol* **11:**1–20.
Samama P, Cotecchia S, Costa T, and Lefkowitz RJ (1993) A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* **268:**4625–4636.
Sealfon SC, Weinstein H, and Millar RP (1997) Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. *Endocr Rev* **18:**180–205.
Shinitzky M, Hazum E, and Fridkin M (1976) Structure-activity relationships of luliberin substituted at position 8. *Biochim Biophys Acta* **453:**553–557.
Wells JA, Powers DB, Bott RR, Graycar TP, and Estell DA (1987) Designing substrate specificity by protein engineering of electrostatic interactions. *Proc Natl Acad Sci USA* **84:**1219–1223.
White RB, Eisen JA, Kasten TL, and Fernald RD (1998) Second gene for gonadotropin-releasing hormone in humans. *Proc Natl Acad Sci USA* **95:**305–309.
Zhao MM, Gaivin RJ, and Perez DM (1998) The third extracellular loop of the beta2-adrenergic receptor can modulate receptor/G protein affinity. *Mol Pharmacol* **53:**524–529.
Zhou W, Rodic V, Kitanovic S, Flanagan CA, Chi L, Weinstein H, Maayani S, Millar RP, and Sealfon SC (1995) A locus of the gonadotropin-releasing hormone receptor that differentiates agonist and antagonist binding sites. *J Biol Chem* **270:**18853–18857.

Address correspondence to: Dr. Colleen A. Flanagan, Division of Medical Biochemistry, University of Cape Town Faculty of Health Sciences, Anzio Road, Observatory, 7925, South Africa. E-mail: flanagan@curie.uct.ac.za