Chimaeric gonadotropin-releasing hormone (GnRH) peptides with improved affinity for the catfish (*Clarias gariepinus*) GnRH receptor

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The gonadotropin-releasing hormone (GnRH) receptor in catfish differs from its mammalian counterparts in showing a very low affinity for the hypothalamic GnRH form [i.e. catfish GnRH (cfGnRH)] and a very high affinity for the highly conserved mesencephalic GnRH, chicken GnRH-II (cGnRH-II). In the present study we investigated the molecular interactions between ligand and receptor involved in determining the ligand selectivity of the catfish GnRH receptor. Studies on the binding characteristics of the catfish GnRH receptor for cfGnRH and cGnRH-II as well as for mammalian GnRH (mGnRH) and synthetic chimaeric GnRHs, differing at positions 5, 7 and 8, revealed that the low affinity of the catfish receptor for cfGnRH can be improved by replacing Leu⁷ by a tryptophan residue and/or Asn⁸ by either a

tyrosine or an arginine residue. Testing cfGnRH and cGnRH-II as well as mGnRH and the chimaeric GnRHs on Asp³⁰⁴ → Ala, Asp³⁰⁴ → Glu and Asp³⁰⁴ → Asn mutant catfish GnRH receptors revealed that Asp³⁰⁴ of the catfish receptor mediates the recognition of Arg⁸ in mGnRH, as well as in the chimaeric peptides [Arg⁸]cfGnRH and [Arg⁸]cGnRH-II, but seems to be less important for the recognition of Tyr⁸ in cGnRH-II. On the basis of these results, a three-dimensional model for the binding of [Arg⁸]cGnRH-II to the catfish GnRH receptor is proposed.

Key words: computational modelling, G-protein-coupled receptor, ligand selectivity, site-directed mutagenesis.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a decapeptide that is essential in the control of reproduction in all vertebrates [1]. Many agonistic and antagonistic analogues have been synthesized and are used clinically for the treatment of GnRH deficiency states, infertility, hormone-dependent cancers, precocious puberty and different gynaecological disorders [2]. So far, 13 different native GnRHs have been characterized from various species [3]. In addition to a hypothalamic GnRH of variable sequence, many vertebrate species, including humans, have been shown to express a second, invariant GnRH form designated chicken GnRH-II (cGnRH-II; [His⁵,Trp⁷,Tyr⁸]GnRH) in the midbrain [4]. The African catfish (*Clarias gariepinus*) has two endogenous ligands: the hypothalamic catfish GnRH (cfGnRH; [His⁵,Leu⁷,Asn⁸]GnRH) and the mesencephalic cGnRH-II [5].

GnRH receptors belong to the family of rhodopsin-like G-protein-coupled receptors (GPCRs). Previous studies have shown that mammalian GnRH receptors are selective for mammalian GnRH (mGnRH), but can also be substantially activated by cGnRH-II [6–8]. Non-mammalian GnRH receptors are promiscuous in interacting well with most of the vertebrate GnRHs but seem to have a preference for cGnRH-II [8,9]. In accordance with this, we have shown that the catfish GnRH receptor has a 1000-fold lower affinity for cfGnRH than for cGnRH-II [10,11] and that the lower biological activity of cfGnRH is compensated for by its higher concentration in the catfish pituitary [10].

It has been shown that the negatively charged Glu³⁰¹ residue of the mouse GnRH receptor has a major role in the receptor recognition of the Arg⁸ residue in mGnRH [6]. In this context it is intriguing that the catfish GnRH receptor contains a negatively charged Asp³⁰⁴ residue at the position similar to Glu³⁰¹ in the mouse receptor, but nevertheless has a low affinity for mGnRH

[12]. Such differences in agonist selectivity between vertebrate GnRH receptors might form the basis for elucidating specific receptor–ligand interactions. This knowledge, in turn, might facilitate the rational design of novel peptide and/or non-peptide GnRH analogues.

In the present study we tried to shed more light on the molecular mechanism involved in the ligand selectivity of the catfish GnRH receptor, with the intention of answering two main questions. First, which residue(s) in the ligand enable the catfish GnRH receptor to bind cGnRH-II with high selectivity? Secondly, what is the function of Asp³⁰⁴ in the catfish GnRH receptor, because the African catfish has no endogenous ligand containing a positively charged amino acid residue at position 8? To answer these questions we performed functional studies with the two endogenous ligands (namely cfGnRH and cGnRH-II) and with mGnRH, as well as various chimaeric GnRH analogues in combination with the site-directed mutagenesis of Asp³⁰⁴ in the catfish GnRH receptor. In line with our experimental findings, we describe molecular models of the catfish GnRH receptor in which endogenous cGnRH-II as well as the potent [Arg⁸]cGnRH-II peptide were docked into the presumed GnRH-binding pocket of the catfish GnRH receptor.

This comparative study exemplifies how structural and functional similarities and differences between GPCRs of evolutionarily distant animal species might help in identifying residues involved in mediating ligand selectivity.

EXPERIMENTAL

Peptides

mGnRH (pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Arg⁸-Pro⁹-Gly¹⁰-NH₂) was purchased from Sigma (St Louis, MO, U.S.A.).

Abbreviations used: GnRH, gonadotropin-releasing hormone; cfGnRH, catfish GnRH; cGnRH-II, chicken GnRH-II; EL, extracellular loop; GPCR, G-protein-coupled receptor; mGnRH, mammalian GnRH; MD, molecular dynamics.

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cGnRH-II ([His⁵,Trp⁷,Tyr⁸]GnRH) and cfGnRH ([His⁵,Leu⁷,Asn⁸]GnRH), as well as [His⁵,Leu⁷,Tyr⁸]GnRH, [His⁵,Trp⁷,Asn⁸]GnRH, [His⁵,Trp⁷,Arg⁸]GnRH, [His⁵,D-Trp⁶,Trp⁷,Arg⁸]GnRH and [His⁵,Leu⁷,Arg⁸]GnRH, were synthesized at the Institute of Molecular Pharmacology (Berlin, Germany). In brief, peptides were synthesized automatically (MilliGen 9050 Peptide Synthesizer) by solid-phase methods with standard fluoren-9-ylmethoxycarbonyl chemistry in continuous-flow mode as described previously for the synthesis of corticotropin-releasing factor analogues [13]. Purification was performed by preparative reverse-phase HPLC to give final products of more than 95 % purity. Characterization of accurate peptide synthesis was achieved by MS, yielding the expected $[M + H]^+$ mass peaks, and by determining the amino acid composition.

Mutant catfish GnRH receptor constructs

Mutations in the catfish GnRH-R cDNA insert [12] were introduced by using the pALTER-1 *in vitro* mutagenesis system (Promega, Madison, WI, U.S.A.) in accordance with the manufacturer's instructions. Asp³⁰⁴ was mutated to an alanine residue, a glutamate residue or an asparagine residue, generating Asp³⁰⁴ → Ala, Asp³⁰⁴ → Glu and Asp³⁰⁴ → Asn mutant catfish GnRH receptor constructs. The mutations introduced in these constructs were confirmed by sequence analysis. The mutant cDNA inserts were then subcloned into pcDNA3 (Invitrogen, San Diego, CA, U.S.A.) for expression studies.

Cell culture and transfection

HEK-293T cells were cultured as described previously [11] and transiently transfected with wild-type or mutant catfish GnRH-R cDNA constructs (5 µg of DNA per 100 mm² dish, or 2.5 µg of DNA per 60 mm² dish) with the SuperFect transfection method (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. To prevent cells from detaching, 24-well and 48-well plates were coated with poly-(D-lysine) (Sigma) before being seeded with cells.

ELISA detection of GnRH receptor expression

HEK-293T cells in 60 mm² plates were transfected with 2.5 µg of wild-type receptor cDNA-pcDNA3 construct (positive control), mutant receptor cDNA-pcDNA3 construct, or pcDNA3 only (negative control). After 24 h, cells were transferred to 24-well plates (5 × 10⁵ cells per well) and, after an additional 24 h, were fixed with 4 % (w/v) paraformaldehyde in PBS at 20 °C for 30 min. Samples were then blocked with 1 % (w/v) non-fat dried milk in 0.1 M NaHCO₃ at 20 °C for 4 h, then incubated overnight at 4 °C with an antiserum raised against the N-terminus of the catfish GnRH receptor [11] [diluted 1:100 in 10 mM Tris/HCl (pH 7.5)/150 mM NaCl containing 0.1 % BSA]. After exposure to peroxidase-conjugated goat anti-rat IgG [diluted 1:1000 in 0.1 M NaHCO₃/1 % non-fat dried milk (Sigma)] at 20 °C for 2 h, peroxidase was detected with TMB liquid substrate system (Sigma) for 30 min. The A_{450} of the negative control was subtracted and the values were expressed as a percentage of the positive control. All constructs were tested in triplicate in three separate experiments.

Receptor binding assay

cGnRH-II (2.5 µg) was iodinated with the chloramine-T method and subsequently purified by C₁₈ column chromatography [11]. The specific radioactivity of the radioligand was 111 µCi/mmol. Ligand-binding assays were performed on cell membranes from receptor-expressing HEK-293T cells as described previously [14].

In brief, purified membranes were incubated in 0.5 ml of assay buffer [40 mM Tris/HCl (pH 7.4)/2 mM MgCl₂/0.1 % BSA] at 4 °C for 2 h with increasing concentrations of ¹²⁵I-labelled cGnRH-II (up to 1330000 d.p.m.) in the presence or absence of 1 µM unlabelled cGnRH-II. For displacement studies, purified membranes were incubated at 4 °C for 2 h with approx. 1 nM ¹²⁵I-labelled cGnRH-II (133000 d.p.m.) in 0.5 ml of assay buffer in the presence of various concentrations of unlabelled native and chimaeric GnRH analogues. The concentration of ¹²⁵I-labelled cGnRH-II approximated its K_d at the wild-type catfish GnRH receptor (2 nM; see the Results section). The membranes were then filtered and the radioactivity was counted. All binding studies were performed in triplicate in three independent experiments. Binding parameters were determined from saturation and displacement curves with the PRISM2 (GraphPad, San Diego, CA, U.S.A.) software package.

Total inositol phosphates

Total inositol phosphates were extracted and separated as described previously [15]. In brief, 24 h after transfection, cells were transferred to 48-well plates [2.5 × 10⁵ cells per well] in 0.5 ml of inositol-free Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) dialysed fetal calf serum] and incubated for 24 h with [³H]inositol (1 µCi/ml; Amersham, Little Chalfont, Bucks., U.K.). Medium was removed and cells were washed and preincubated for 10 min with assay medium (Hepes-modified DMEM containing 10 mM LiCl). Various concentrations of different native and chimaeric GnRH analogues were added at 37 °C for 45 min; the assay medium was then aspirated. After extraction with 10 mM formic acid at 4 °C for at least 90 min, extracts were transferred to columns containing Dowex (AG 1X8-200) anion-exchange resin (Sigma). Total inositol phosphates were then eluted and the amount of radioactivity was counted. Assays were performed in duplicate in three separate experiments. EC₅₀ values were determined from concentration-response curves by using PRISM2.

Statistical analysis

All results are presented as means ± S.E.M. for three independent experiments. Statistical analysis was performed with a one-way analysis of variance and, where $P < 0.05$, was followed by the Bonferroni test. $P < 0.05$ was considered to be significant.

Computational methods

The catfish GnRH receptor model was based on Baldwin's template of the electron-density structure of bovine rhodopsin [16] and built from an existing human GnRH receptor model [17] by substituting the appropriate amino acids in the transmembrane regions with the program SYBYL 6.4. Intracellular loops 1 and 3, extracellular loop 2 (EL 2) and the intracellular C-terminus were rebuilt with the use of the loop search option in SYBYL 6.4. By analogy with the human GnRH receptor [18], one disulphide bridge (Cys¹¹⁷-Cys¹⁹⁴) was included in the model. Start coordinates of the docked GnRH peptides cGnRH-II and [His⁵,Trp⁷,Arg⁸]GnRH were also abstracted from the human GnRH receptor model and in accordance with existing experimental site-directed mutagenesis data on that receptor [17]. The catfish GnRH receptor complexes were minimized with AMBER 4.1 FF before running a molecular dynamics (MD) simulation of 500 ps *in vacuo* [time step = 2 fs, $T = 300$ K, $\epsilon = 1$, and using a cut-off for non-bonded interactions of 9 Å (0.9 nm)]. For representation, the frame with the lowest interaction enthalpy ($\Delta H_{\text{interaction}} = \Delta H_{\text{complex}} - \Delta H_{\text{receptor}} - \Delta H_{\text{ligand}}$) was selected out of 500 frames (every 1 ps) generated.

RESULTS

Activities of cGnRH-II analogues with and without Trp⁷ and/or Tyr⁸

From previous work we knew that the catfish GnRH receptor expressed in HEK-293T cells has an approx. 1000-fold higher affinity for cGnRH-II than for cfGnRH [11]. In addition, cGnRH-II is approx. 1000-fold more potent than cfGnRH in stimulating second messenger production in these cells [11]. In the present study we confirmed that the catfish GnRH receptor has high-affinity binding characteristics for cGnRH-II, while the K_i value of the receptor for cfGnRH was approx. 1083-fold higher (Figure 1A and Table 1).

The catfish GnRH receptor binds cGnRH-II, from all GnRHs tested, with the highest affinity. To study which amino acid residue(s) in cGnRH-II are important to enable the catfish GnRH receptor to bind cGnRH-II with this high affinity, we created two chimaeric GnRH analogues, namely [His⁵,Leu⁷,Tyr⁸]GnRH and [His⁵,Trp⁷,Asn⁸]GnRH. These analogues represent two intermediate peptides of the two endogenous African catfish GnRH ligands cGnRH-II and cfGnRH [5], which differ from each other at positions 7 and 8 (Table 1). Surprisingly, the affinity of the receptor for these cGnRH-II analogues was decreased significantly compared with that for cGnRH-II (52-fold and 15-fold higher K_i values for [His⁵,Leu⁷,Tyr⁸]GnRH and [His⁵,Trp⁷,Asn⁸]GnRH respectively; $P < 0.001$) (Figure 1A and Table 1).

In addition to the binding studies, we also compared the ability of cGnRH-II with that of the cGnRH-II analogues containing substitutions on position 7 and/or position 8 to stimulate phospholipase C activity in HEK-293T cells expressing the catfish GnRH receptor. After 24 h of labelling with [³H]inositol and 45 min of stimulation with various concentrations of the different GnRH peptides, the cellular accumulation of total [³H]inositol phosphates was determined. The signal-transduction data reflected the results from the binding studies. Thus the high potency of cGnRH-II was diminished significantly by replacing amino acid residues at either position 7 or position 8 or both (93-fold, 76-fold and 976-fold higher EC_{50} values for [His⁵,Leu⁷,Tyr⁸]GnRH, [His⁵,Trp⁷,Asn⁸]GnRH and [His⁵,Leu⁷,Asn⁸]GnRH (cfGnRH) respectively; $P < 0.001$) (Figure 1B and Table 1).

Activities of GnRH analogues with and without Arg⁸

The arginine residue on position 8 of mGnRH is necessary for the mouse GnRH receptor to bind mGnRH with high affinity [6].

Table 1 Summary of ligand binding and [³H]inositol phosphate production of HEK-293T cells expressing wild-type catfish GnRH receptor by native and chimaeric forms of GnRH

The pK_i values were obtained from displacement studies of binding of [¹²⁵I]-cGnRH-II to membranes prepared from HEK-293T cells transiently expressing the wild-type GnRH receptor. The pEC_{50} values were obtained from total [³H]inositol phosphate studies as described in the Experimental section. Results are means $\pm$ S.E.M. for three independent experiments; means in the same column that are significantly different are indicated by different superscript letters. Abbreviation: n.d., not determined.

Peptide	pK_i	pEC_{50}
Native peptides		
[His ⁵ ,Leu ⁷ ,Asn ⁸]GnRH (cfGnRH)	6.09 ± 0.13^a	5.91 ± 0.16^a
[Tyr ⁵ ,Leu ⁷ ,Arg ⁸]GnRH (mGnRH)	6.18 ± 0.04^a	n.d.
[His ⁵ ,Trp ⁷ ,Tyr ⁸]GnRH (cGnRH-II)	9.12 ± 0.09^b	8.90 ± 0.17^f
Chimaeric peptides		
[His ⁵ ,Leu ⁷ ,Arg ⁸]GnRH	7.01 ± 0.13^c	n.d.
[His ⁵ ,Leu ⁷ ,Tyr ⁸]GnRH	7.41 ± 0.06^c	6.93 ± 0.03^g
[His ⁵ ,Trp ⁷ ,Asn ⁸]GnRH	7.95 ± 0.07^d	7.01 ± 0.07^g
[His ⁵ ,Trp ⁷ ,Arg ⁸]GnRH	9.27 ± 0.02^b	n.d.
[His ⁵ ,p-Trp ⁶ ,Trp ⁷ ,Arg ⁸]GnRH	9.41 ± 0.09^b	n.d.

Table 2 Alignment of the amino acid sequences in EL 3 of selected members of the GnRH receptor family

Glu³⁰¹ of the mouse GnRH receptor and the Asp/Glu residues at the similar position in other mammalian and fish GnRH receptors, including Asp³⁰⁴ of the catfish GnRH receptor, are shown in bold.

Receptor	EL 3
Mouse GnRH receptor	WYWFDPPEMLNRVS E PVNH
Human GnRH receptor	WYWFDPPEMLNRLS D PVNH
Rat GnRH receptor	WYWFDPPEMLNRVS E PVNH
Catfish GnRH receptor	WYWFQPEMLHVIP D YVHH
Goldfish GnRH receptor A	WYWFQPEMLKVTP E YIHH
Goldfish GnRH receptor B	WYWFQPRMLQSMF E YIHH

However, the catfish GnRH receptor has only a poor affinity for mGnRH [11], although the receptor contains negatively charged Asp³⁰⁴ at a position similar to Glu³⁰¹ in the mouse GnRH receptor (Table 2). To obtain further insight into the specific interaction between GnRH receptor and GnRH ligand, we synthesized [Arg⁸]cfGnRH and [Arg⁸]cGnRH-II analogues

Figure 1 Activities of cGnRH-II analogues with and without Trp⁷ and/or Tyr⁸

Binding of [¹²⁵I]-cGnRH-II (A) and [³H]inositol phosphate ([³H]-IP) production (B) in the presence of various concentrations of [His⁵,Trp⁷,Tyr⁸]GnRH (cGnRH-II, ●), [His⁵,Leu⁷,Asn⁸]GnRH (cfGnRH, □), the two intermediate GnRH peptides, [His⁵,Leu⁷,Tyr⁸]GnRH (■) and [His⁵,Trp⁷,Asn⁸]GnRH (○), of HEK-293T cells transiently expressing the wild-type catfish GnRH receptor were measured as described in the Experimental section. Results are means $\pm$ S.E.M. for triplicate observations from a single representative experiment.

Figure 2 Ligand binding of $[\text{His}^5, \text{Leu}^7, \text{Asn}^8]\text{GnRH}$ (cfGnRH, $\square$) and $[\text{His}^5, \text{Leu}^7, \text{Arg}^8]\text{GnRH}$ ($\blacksquare$) to membranes prepared from HEK-293T cells transiently expressing wild-type (A), $\text{Asp}^{304} \rightarrow \text{Ala}$ mutant (B), $\text{Asp}^{304} \rightarrow \text{Asn}$ mutant (C) or $\text{Asp}^{304} \rightarrow \text{Glu}$ mutant (D) catfish GnRH receptor

Displacement of ^{125}I -cGnRH-II binding to membranes prepared from HEK-293T cells transiently expressing either wild-type catfish GnRH receptor or one of the mutant catfish GnRH receptors by various concentrations of cfGnRH or $[\text{His}^5, \text{Leu}^7, \text{Arg}^8]\text{GnRH}$ were measured as described in the Experimental section. Results are means $\pm$ S.E.M. for triplicate observations from a single representative competition binding experiment.

Figure 3 Ligand binding of $[\text{His}^5, \text{Trp}^7, \text{Tyr}^8]\text{GnRH}$ (cGnRH-II, $\square$) and $[\text{His}^5, \text{Trp}^7, \text{Arg}^8]\text{GnRH}$ ($\blacksquare$) to membranes prepared from HEK-293T cells transiently expressing wild-type (A) or $\text{Asp}^{304} \rightarrow \text{Ala}$ mutant (B) catfish GnRH receptor

Displacement of ^{125}I -cGnRH-II binding to membranes prepared from HEK-293T cells transiently expressing either wild-type catfish GnRH receptor or the $\text{Asp}^{304} \rightarrow \text{Ala}$ mutant catfish GnRH receptor by various concentrations of cGnRH-II or $[\text{His}^5, \text{Trp}^7, \text{Arg}^8]\text{GnRH}$ were measured as described in the Experimental section. Results are means $\pm$ S.E.M. for triplicate observations from a single representative competition binding experiment.

(namely $[\text{His}^5, \text{Leu}^7, \text{Arg}^8]\text{GnRH}$ and $[\text{His}^5, \text{Trp}^7, \text{Arg}^8]\text{GnRH}$ respectively). The catfish GnRH receptor had a higher affinity for $[\text{His}^5, \text{Leu}^7, \text{Arg}^8]\text{GnRH}$ than for $[\text{His}^5, \text{Leu}^7, \text{Asn}^8]\text{GnRH}$ (cfGnRH) and $[\text{Tyr}^5, \text{Leu}^7, \text{Arg}^8]\text{GnRH}$ (mGnRH) ($P < 0.001$; Table 1 and Figure 2A). Furthermore, the catfish GnRH receptor showed a K_i for the $[\text{His}^5, \text{Trp}^7, \text{Arg}^8]\text{GnRH}$ peptide similar to that for $[\text{His}^5, \text{Trp}^7, \text{Tyr}^8]\text{GnRH}$ (cGnRH-II) ($P > 0.05$; Table 1 and Figure 3A).

In addition, a conformationally constrained $[\text{D-Trp}^6, \text{Arg}^8]\text{cGnRH-II}$ analogue ($[\text{His}^5, \text{D-Trp}^6, \text{Trp}^7, \text{Arg}^8]\text{GnRH}$) was used to investigate whether the catfish GnRH receptor binds cGnRH-II in a constrained conformation, analogous to the binding mode

of mGnRH to mammalian GnRH receptors [8]. The affinity of the catfish GnRH receptor for this constrained peptide was similar to that for cGnRH-II and $[\text{His}^5, \text{Trp}^7, \text{Arg}^8]\text{GnRH}$ ($P > 0.05$; Table 1).

Characteristics of $\text{Asp}^{304} \rightarrow \text{Ala}$, $\text{Asp}^{304} \rightarrow \text{Glu}$ and $\text{Asp}^{304} \rightarrow \text{Asn}$ mutant catfish GnRH receptors

Next we investigated the role of the Asp^{304} residue in EL 3 of the catfish GnRH receptor. To obtain further insight into the GnRH-selectivity characteristics of the catfish GnRH receptor, mediated by the presumed interaction of Asp^{304} of the receptor with

Table 3 Characteristics of wild-type and mutant catfish GnRH receptors transiently expressed in HEK-293T cells

The cell-surface expression of wild-type and mutant receptor constructs, transiently transfected in HEK-293T cells, was measured in an ELISA with an antibody raised against the N-terminal part of the catfish GnRH receptor. $B_{\max}$ and pK_d values were obtained from saturation studies of [125 I]-cGnRH-II binding to membranes prepared from HEK-293T cells transiently expressing wild-type or mutant GnRH receptors. The agonist-induced accumulation of [3 H]inositol phosphates was determined in HEK-293T cells transiently expressing wild-type and mutant GnRH receptors after stimulation for 45 min with 10 nM cGnRH-II as described in the Experimental section. Results are means $\pm$ S.E.M. for three independent experiments. * $P < 0.05$ compared with the wild-type receptor. Abbreviation: IP, inositol phosphate.

Parameter	Wild-type	Asp ³⁰⁴ → Ala	Asp ³⁰⁴ → Glu	Asp ³⁰⁴ → Asn
ELISA-detected expression (% of wild-type)	100	68 $\pm$ 8*	110 $\pm$ 4	80 $\pm$ 3
$B_{\max}$ (pmol/mg of protein)	10.4 $\pm$ 1.7	6.3 $\pm$ 2.0	10.7 $\pm$ 1.8	9.0 $\pm$ 2.4
pK_d	8.75 $\pm$ 0.11	8.53 $\pm$ 0.19	8.58 $\pm$ 0.12	8.56 $\pm$ 0.09
IP response (% of wild-type)	100	81 $\pm$ 7	110 $\pm$ 7	86 $\pm$ 7

Figure 4 Ligand binding of [Tyr⁵,Leu⁷,Arg⁸]GnRH (mGnRH) to membranes prepared from HEK-293T cells transiently expressing wild-type (■), Asp³⁰⁴ → Ala mutant (○), Asp³⁰⁴ → Glu mutant (□) or Asp³⁰⁴ → Asn mutant (●) catfish GnRH receptor

Displacement of [125 I]-cGnRH-II binding to membranes prepared from HEK-293T cells transiently expressing either wild-type catfish GnRH receptor or one of the mutant catfish GnRH receptors by various concentrations of mGnRH were measured as described in the Experimental section. Results are means $\pm$ S.E.M. for triplicate observations from a single representative competition binding experiment.

residue 8 of the various GnRHs and GnRH analogues, we generated different mutant catfish GnRH receptors in which we replaced Asp³⁰⁴ by an alanine, glutamic acid or asparagine residue. Table 3 summarizes the data on the cell-surface expression of these mutant receptor proteins, the binding of [125 I]-cGnRH-II to them, and their ability to activate phospholipase C. Except for a slightly lower expression level of the Asp³⁰⁴ → Ala mutant catfish GnRH receptor ($P < 0.05$; Table 3), all other characteristics of the Asp³⁰⁴ → Ala, Asp³⁰⁴ → Glu and Asp³⁰⁴ → Asn mutant catfish GnRH receptors were comparable with those of the wild-type receptor ($P > 0.05$; Table 3).

Asp³⁰⁴ of the catfish GnRH receptor is important in the recognition of [Arg⁸]GnRH peptides

In an initial binding study we tested whether the affinity of the catfish GnRH receptor for [Tyr⁵,Leu⁷,Arg⁸]GnRH (mGnRH) decreased when Asp³⁰⁴ was replaced by an alanine, glutamic or asparagine residue. Figure 4 shows that the Asp³⁰⁴ → Glu mutant receptor, in which Asp was replaced by another negatively charged residue, had an affinity for mGnRH that was not significantly different from that of the wild-type receptor ($P > 0.05$) (Table 4). However, replacement of Asp³⁰⁴ by an uncharged residue (Asp³⁰⁴ → Ala and Asp³⁰⁴ → Asn mutant receptors) resulted in a decreased affinity for mGnRH ($P < 0.001$ and $P < 0.01$ respectively) (Table 4 and Figure 4).

To substantiate these results we investigated whether the Asp³⁰⁴ → Glu, Asp³⁰⁴ → Ala and Asp³⁰⁴ → Asn mutant catfish GnRH receptors were able to discriminate between cfGnRH and the [Arg⁸]cfGnRH peptide, like the wild-type receptor (see above). The Asp³⁰⁴ → Ala and Asp³⁰⁴ → Asn mutant receptors had similar K_i values for [His⁵,Leu⁷,Asn⁸]GnRH (cfGnRH) and [His⁵,Leu⁷,Arg⁸]GnRH ($P > 0.05$) (Figures 2B and 2C, and Table 4). In contrast, the Asp³⁰⁴ → Glu mutant receptor showed a higher affinity for [His⁵,Leu⁷,Arg⁸]GnRH than for cfGnRH ($P < 0.05$) (Figure 2D and Table 4), although the difference in affinities was less pronounced for the Asp³⁰⁴ → Glu mutant receptor than for the wild-type receptor.

In addition, we determined the affinity of the Asp³⁰⁴ → Ala, Asp³⁰⁴ → Glu and Asp³⁰⁴ → Asn mutant catfish GnRH receptors for cGnRH-II and [Arg⁸]cGnRH-II. The Asp³⁰⁴ → Glu mutant receptor showed no significant difference in affinity for either [His⁵,Trp⁷,Tyr⁸]GnRH (cGnRH-II) or [His⁵,Trp⁷,Arg⁸]GnRH ($P > 0.05$) (Table 4), in accord with our findings on the wild-type receptor (see above). In contrast, the Asp³⁰⁴ → Ala and Asp³⁰⁴ →

Table 4 Summary of the affinities of the Asp³⁰⁴ → Ala, Asp³⁰⁴ → Glu and Asp³⁰⁴ → Asn mutant catfish GnRH receptors for various native and chimaeric GnRH peptides in comparison with the wild-type GnRH receptor

The pK_i values were obtained from displacement studies of [125 I]-cGnRH-II binding to membranes prepared from HEK-293T cells transiently expressing wild-type or mutant GnRH receptors. Results are means $\pm$ S.E.M. for three independent experiments. * $P < 0.05$ compared with the wild-type receptor.

pK_i	Wild-type	Asp ³⁰⁴ → Ala	Asp ³⁰⁴ → Glu	Asp ³⁰⁴ → Asn
Native peptides				
[His ⁵ ,Leu ⁷ ,Asn ⁸]GnRH (cfGnRH)	6.09 $\pm$ 0.13	5.95 $\pm$ 0.13	5.86 $\pm$ 0.10	6.02 $\pm$ 0.01
[Tyr ⁵ ,Leu ⁷ ,Arg ⁸]GnRH (mGnRH)	6.18 $\pm$ 0.04	4.98 $\pm$ 0.28*	5.85 $\pm$ 0.17	5.51 $\pm$ 0.06*
[His ⁵ ,Trp ⁷ ,Tyr ⁸]GnRH (cGnRH-II)	9.12 $\pm$ 0.09	8.72 $\pm$ 0.10	9.12 $\pm$ 0.11	9.04 $\pm$ 0.03
Chimaeric peptides				
[His ⁵ ,Leu ⁷ ,Arg ⁸]GnRH	7.01 $\pm$ 0.13	5.84 $\pm$ 0.08*	6.45 $\pm$ 0.06*	5.99 $\pm$ 0.07*
[His ⁵ ,Leu ⁷ ,Tyr ⁸]GnRH	7.41 $\pm$ 0.06	6.91 $\pm$ 0.07*	7.43 $\pm$ 0.03	6.95 $\pm$ 0.09*
[His ⁵ ,Trp ⁷ ,Asn ⁸]GnRH	7.95 $\pm$ 0.07	7.68 $\pm$ 0.12	7.83 $\pm$ 0.07	7.91 $\pm$ 0.03
[His ⁵ ,Trp ⁷ ,Arg ⁸]GnRH	9.27 $\pm$ 0.02	7.99 $\pm$ 0.10*	8.65 $\pm$ 0.01*	8.29 $\pm$ 0.06*
[His ⁵ ,D-Trp ⁶ ,Trp ⁷ ,Arg ⁸]GnRH	9.41 $\pm$ 0.09	8.35 $\pm$ 0.20*	9.01 $\pm$ 0.11	8.57 $\pm$ 0.08*

Figure 5 Molecular model of the catfish GnRH receptor with [His⁵,Trp⁷,Arg⁸]GnRH docked into the binding pocket

(A) The green tube represents catfish GnRH receptor backbone, capped sticks with orange carbon atoms represent [His⁵,Trp⁷,Arg⁸]GnRH and capped sticks with white carbon atoms represent receptor side chains. (B) Detail of the molecular model of the binding of [His⁵,Trp⁷,Arg⁸]GnRH to the catfish GnRH receptor. Arg⁸ makes two hydrogen bonds with Asp³⁰⁴, Gly⁶ is near the entrance of the receptor and permits a β -turn-type conformation of the agonist, the N-terminal pGlu¹ interacts with Lys¹²⁴, and the C-terminal Gly¹⁰-NH₂ hydrogen bonds with Asn¹⁰⁵ in EL1 in agreement with experimental results (see the text for details).

Asn mutant receptor constructs had a lower affinity for [His⁵, Trp⁷, Arg⁸]GnRH than for cGnRH-II ($P < 0.01$ and $P < 0.001$ respectively) (Figure 3B and Table 4). In addition, the conformationally constrained [His⁵, D-Trp⁶, Trp⁷, Arg⁸]GnRH peptide reflected the findings for the [His⁵, Trp⁷, Arg⁸]GnRH analogue (Table 4).

Asp³⁰⁴ of the catfish GnRH receptor is not essential for the recognition of [Tyr⁸]GnRH peptides

To test whether Tyr⁸ of cGnRH-II was able to interact with Asp³⁰⁴ of the catfish GnRH receptor, we studied whether the Asp³⁰⁴ → Ala, Asp³⁰⁴ → Glu and Asp³⁰⁴ → Asn mutant catfish GnRH receptors were able to discriminate between [His⁵, Trp⁷, Tyr⁸]GnRH (cGnRH-II) and [His⁵, Trp⁷, Asn⁸]GnRH, and between [His⁵, Leu⁷, Tyr⁸]GnRH and [His⁵, Leu⁷, Asn⁸]GnRH (cfGnRH). Ligand binding studies on wild-type and Asp³⁰⁴-mutant receptors revealed that these receptors had similar affinities for cGnRH-II ($P > 0.05$) (Table 4) and that the Asp³⁰⁴-mutant receptors did discriminate between [His⁵, Trp⁷, Tyr⁸]GnRH (cGnRH-II) and [His⁵, Trp⁷, Asn⁸]GnRH, similarly to the wild-type receptor ($P < 0.001$) (Table 4). In contrast, Asp³⁰⁴ → Ala and Asp³⁰⁴ → Asn mutant catfish GnRH receptors had a lower affinity for [His⁵, Leu⁷, Tyr⁸]GnRH than for the wild-type and the Asp³⁰⁴ → Glu mutant receptors ($P < 0.01$) (Table 4). Nevertheless, all Asp³⁰⁴-mutant receptors could still discriminate between [His⁵, Leu⁷, Tyr⁸]GnRH and [His⁵, Leu⁷, Asn⁸]GnRH ($P < 0.001$) (Table 4).

Computational modelling

On the basis of the above findings, we developed models of the catfish GnRH receptor with either [His⁵, Trp⁷, Arg⁸]GnRH (Figure 5) or [His⁵, Trp⁷, Tyr⁸]GnRH (cGnRH-II; results not shown) docked into the catfish GnRH receptor-binding pocket. These models were built in agreement with published data from mutagenesis studies on mammalian GnRH receptors [17]. For example, experimental results suggest that the C-terminal Gly¹⁰-NH₂ of GnRH interacts with Asn¹⁰² of the human GnRH receptor [7]. Photoaffinity labelling studies also identified the side chain of [D-Trp⁶]GnRH in the proximity of Cys¹⁴ of the receptor, thus accommodating the ligand in the region between the ELs. Moreover, mutation of Lys¹²¹ to Gln affects GnRH agonist binding but not antagonist binding to the human GnRH receptor [19]. In our previous GnRH receptor model we proposed that the hydrogen-bond donor of Lys¹²¹ is in contact with the N-terminal pGlu¹ residue of GnRH, which is lacking in peptide antagonists [17]. The present molecular models of the catfish GnRH receptor obtained from 500 ps of MD simulations are also in accord with these data.

According to our model, in which [His⁵, Trp⁷, Arg⁸]GnRH was docked into the catfish GnRH receptor-binding pocket, the positively charged Arg⁸ of [His⁵, Trp⁷, Arg⁸]GnRH makes strong ionic hydrogen bonds with the negatively charged Asp³⁰⁴ (Figure 5B). In addition, MD simulations, docking [His⁵, Trp⁷, Tyr⁸]GnRH (cGnRH-II) into the catfish GnRH receptor-binding pocket, revealed that Tyr⁸ is able to form a hydrogen bond with Asp³⁰⁴ in the wild-type catfish receptor (results not shown). Additional MD simulations of cGnRH-II, now docked into the binding pocket of the Asp³⁰⁴ → Ala mutant catfish receptor, showed that, in the absence of the negatively charged side chain of the Asp³⁰⁴ residue in the receptor, Tyr⁸ of cGnRH-II can also make a hydrogen bond with the backbone carbonyl group of Ala¹⁹⁵ in this receptor (results not shown). The aromatic ring of Tyr⁸ seems to be embedded in a hydrophobic pocket formed by the aromatic rings of His³⁰⁷ and Trp¹⁰⁴ of the receptor.

DISCUSSION

In the present study we used the catfish GnRH receptor as a model to investigate which amino acid residues in GnRHs are important to enable the receptor to bind cGnRH-II with the highest affinity. In agreement with previous studies, we demonstrated that the catfish GnRH receptor has a higher affinity for cGnRH-II than for cfGnRH and mGnRH [11,12], and that cGnRH-II has a higher potency than cfGnRH for second messenger production in HEK-293T cells expressing the catfish GnRH receptor. In the African catfish pituitary, the lower affinity of the catfish GnRH receptor for cfGnRH than for cGnRH-II is compensated for by an approx. 700-fold higher concentration of cfGnRH [10]. It has also been reported that cGnRH-II is the most potent endogenous ligand for the chicken GnRH receptor and the two goldfish GnRH receptors [9,20]. Moreover, apart from the highly potent mGnRH, cGnRH-II is also able to stimulate the mouse and human GnRH receptors to accumulate inositol phosphates with an EC₅₀ value in the nanomolar range, comparable with the EC₅₀ value of cGnRH-II for the catfish GnRH receptor [6,7].

His⁵, Trp⁷ and Tyr/Arg⁸ are important residues in the GnRH ligand, allowing the catfish GnRH receptor to bind GnRH with high affinity

The fact that the catfish GnRH receptor has a lower affinity for [Tyr⁵, Leu⁷, Arg⁸]GnRH (mGnRH) than for [His⁵, Leu⁷, Arg⁸]GnRH, which differs from mGnRH only at position 5, demonstrates that the presence of His⁵ in the ligand is needed for the high-affinity interaction of the catfish GnRH receptor with GnRH ligands. Chicken, *Xenopus laevis* and goldfish (*Carassius auratus*) GnRH receptors differ in this respect, because [His⁵]mGnRH resulted in a marked decline in their activity, whereas mammalian GnRH receptors showed similar or higher affinities for [His⁵]mGnRH compared with mGnRH [9,21].

In addition, we identified the Trp⁷ and Tyr⁸ residues as important residues in the GnRH ligand that contribute to the high affinity of the catfish GnRH receptor for cGnRH-II and thus confer on the cGnRH-II ligand its high potency towards the catfish receptor. The fact that the catfish GnRH receptor prefers a GnRH ligand with Trp⁷ rather than Leu⁷ is supported by the finding that this receptor showed a higher affinity for [His⁵, Trp⁷, Tyr⁸]GnRH and [His⁵, Trp⁷, Arg⁸]GnRH than for [His⁵, Leu⁷, Tyr⁸]GnRH and [His⁵, Leu⁷, Arg⁸]GnRH. In this respect, the catfish GnRH receptor is similar to the chicken GnRH receptor but different from mammalian GnRH receptors, which tolerate rather than favour a tryptophan residue at position 7 in the GnRH ligand [9].

Surprisingly, we found that the catfish GnRH receptor has a similar affinity for [His⁵, Trp⁷, Arg⁸]GnRH to that for [His⁵, Trp⁷, Tyr⁸]GnRH (cGnRH-II) but the receptor has a lower K_i for [His⁵, Leu⁷, Arg⁸]GnRH than for [His⁵, Leu⁷, Asn⁸]GnRH (cfGnRH). From these findings we conclude that the catfish GnRH receptor recognizes GnRHs with an arginine residue at position 8 equally as well as GnRHs with a tyrosine residue at position 8, and that GnRHs having an arginine or tyrosine residue at position 8 interact better with the catfish GnRH receptor than GnRHs with an asparagine residue at position 8.

Mammalian GnRH receptors have been reported to prefer a constrained β II-type turn conformation of mGnRH, which is favoured by amino acid substitutions in the D-configuration at position 6 in the ligand [8]. Amino acids at this position can be replaced by large polar D-amino acids, suggesting that these side chains point to the extracellular surroundings of the receptor

protein [17]. The fact that the catfish GnRH receptor had a similar high affinity for [His⁵,D-Trp⁶,Trp⁷,Arg⁸]GnRH, with a bulky tryptophan side chain in the D-configuration at position 6, to that for [His⁵,Trp⁷,Tyr⁸]GnRH (cGnRH-II) and [His⁵,Trp⁷,Arg⁸]GnRH suggests that the catfish GnRH receptor also binds cGnRH-II in a constrained β II-type conformation. We therefore consider the binding modes of the catfish and mammalian GnRH receptors to be similar.

Asp³⁰⁴ of the catfish GnRH receptor mediates the recognition of [Arg⁸]GnRHs but is not essential for the recognition of [Tyr⁸]GnRHs

Non-mammalian GnRHs differ from mGnRH by the substitution of a neutral amino acid for the positively charged arginine residue at position 8. For that reason, non-mammalian GnRH receptors were not expected to contain a negatively charged residue at the position similar to Glu³⁰¹ of the mouse GnRH receptor that confers the high affinity of the mouse receptor for [Arg⁸]GnRH ligands [6]. However, the catfish GnRH receptor contains an aspartic acid residue at position 304, similar to Glu³⁰¹ of the mouse GnRH receptor [12]. We therefore wished to investigate the function of Asp³⁰⁴ in the catfish receptor. Replacement of the negatively charged Asp³⁰⁴ residue with uncharged (alanine or asparagine) residues abolished the preference of this receptor for ligands with Arg⁸, demonstrating that Asp³⁰⁴ of the catfish GnRH receptor is able to mediate the recognition of [Arg⁸]GnRH peptides. Because the conserved Asp/Glu residue in EL 3 is preceded by a proline residue in the catfish receptor compared with a serine residue in mammalian GnRH receptors, such an interaction in non-mammalian GnRH receptors had previously been doubted [21].

Our three-dimensional model with [His⁵,Trp⁷,Arg⁸]GnRH docked into the ligand-binding pocket of the catfish GnRH receptor confirms that the orientation of the acidic side chain of Asp³⁰⁴ in the catfish receptor is such that it can make strong ionic hydrogen bonds with Arg⁸ residues in GnRH ligands. This is in accordance with the experimental results in this study, namely that the mutation of Asp³⁰⁴ in the catfish GnRH receptor to a neutral alanine residue resulted in a more-than-10-fold decrease in affinity for all four agonists (namely mGnRH, [His⁵,Leu⁷,Arg⁸]GnRH, [His⁵,Trp⁷,Arg⁸]GnRH and [His⁵,D-Trp⁶,Trp⁷,Arg⁸]GnRH), which contain a positively charged arginine residue on position 8. In contrast, mutation of Asp³⁰⁴ in the catfish GnRH receptor to the negatively charged Glu residue had a less marked effect on the affinity of the receptor for these [Arg⁸]GnRH peptides. Moreover, the observed interaction of Arg⁸ in [His⁵,Trp⁷,Arg⁸]GnRH with Asp³⁰⁴ of the catfish GnRH receptor is analogous to the binding of Arg⁸ in mGnRH to the aspartic or glutamic residues present at the similar position in human and mouse GnRH receptors respectively [6,17].

Thus the poor affinity of the catfish GnRH receptor for mGnRH is not due to a lack of specific recognition of Arg⁸ in the ligand. Instead, the side chains of the amino acid residues on position 5 (tyrosine) and/or position 7 (leucine) of mGnRH are likely to fit better in mammalian GnRH receptors than in the catfish GnRH receptor.

We reported previously that a model of [D-Trp⁶]mGnRH binding to the human GnRH receptor favours a hydrogen-bond interaction between the hydroxy group of Tyr⁵ in the ligand and Asp²⁹³ in the receptor [17]. The residue in a similar position in the catfish GnRH receptor is Gln²⁹⁵. The model of cGnRH-II binding to the catfish GnRH receptor supports a close proximity between His⁵ and Gln²⁹⁵ but also between His⁵ and His³⁰⁷ (Asn³⁰⁵ in the human receptor). Future studies will be aimed at investi-

gating these predicted interactions. According to our models, the position of residue 7 of cGnRH-II is predicted to be located in a hydrophobic pocket formed by the residues of transmembrane regions 3, 4 and 7 together with residues of EL 2. This is in accordance with the localization of residue 7 of [D-Trp⁶]mGnRH in the human GnRH receptor model [17].

Because the African catfish does not contain endogenous GnRH ligands with an arginine residue at position 8, we hypothesized that Asp³⁰⁴ of the catfish GnRH receptor might be important for the recognition of Tyr⁸ in the endogenous cGnRH-II ligand. Indeed, the molecular model with cGnRH-II docked into the ligand-binding pocket of the catfish GnRH receptor predicts that Tyr⁸ is able to hydrogen bond with Asp³⁰⁴ of the receptor. However, our experimental results suggest only a small contribution of Asp³⁰⁴ to the binding of Tyr⁸-containing agonists. The Glu³⁰¹ → Gln mutant mouse GnRH receptor shares characteristics with the Asp³⁰⁴ → Asn mutant catfish GnRH receptor in that the high affinity of the mouse GnRH receptor for cGnRH-II was not influenced by this mutation [6]. The molecular model for the binding of cGnRH-II to the Asp³⁰⁴ → Ala mutant catfish GnRH receptor demonstrates that, in the absence of the negatively charged side chain of Asp³⁰⁴, Tyr⁸ of cGnRH-II makes a hydrogen bond with Ala¹⁹⁵ in EL 2 of the catfish receptor. Moreover, the aromatic residues His³⁰⁷ and Trp¹⁰⁴ (Asn³⁰⁵ and Trp¹⁰² in the human GnRH receptor) also might contribute to the hydrophobic pocket contacting the aromatic ring of Tyr⁸. Future mutagenesis studies will test the role of these residues of the catfish GnRH receptor on the binding of cGnRH-II.

Our experimental findings have demonstrated that Asp³⁰⁴ of the catfish GnRH receptor is unlikely to be the sole residue that determines the high-affinity binding of [Tyr⁸]GnRH peptides. However, Asp³⁰⁴ of the catfish GnRH receptor does clearly contribute to the recognition of [Arg⁸]GnRH peptides.

In conclusion, the differences in agonist selectivity between non-mammalian and mammalian GnRH receptors in combination with computational three-dimensional molecular models of the receptor–ligand complexes might help us to unravel the molecular mechanisms involved in ligand selectivity. The GnRH receptor might therefore serve as a model for GPCRs in general, demonstrating the usefulness of comparative studies between receptors of evolutionarily distant animal species for the understanding of the mechanism underlying ligand recognition.

We thank Dr G.B. Willars for comments on the manuscript.

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Received 14 May 2001/4 October 2001; accepted 7 November 2001