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Functional analysis of GnRH receptor ligand binding using biotinylated GnRH derivatives

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Abstract

The objective of this study was to determine whether the gonadotrophin-releasing hormone (GnRH) ligand binds to the GnRH receptor (GnRH-R) with either the N- and C-termini or the β-II turn pointing towards the cell. The functionality of GnRH and two biotinylated GnRH derivatives, biotin [D-Lys^6]GnRH and biotin [Gln^1]GnRH biotinylated at positions 6 and 1, respectively was assessed. Streptavidin was also used in combination with these peptides to investigate the effects of the steric hindrance caused by this molecule on ligand binding when bound to the biotin molecules at the two positions. GnRH bound to the receptor with high affinity, which was not affected by the addition of streptavidin. Both the biotinylated derivatives bound to the receptor though with lower affinities than GnRH. The biotin [D-Lys^6]GnRH–streptavidin complex bound to the receptor albeit with lower affinity compared to biotin [D-Lys^6]GnRH only, although it maintained its ability to cause receptor internalisation. The ability of the biotin [Gln^1]GnRH to bind to the receptor was abolished in the presence of excess streptavidin. Both GnRH and biotin [D-Lys^6]GnRH stimulated total inositol phosphate production whereas biotin [Gln^1]GnRH exhibited GnRH antagonist activity. It appears that the small biotin molecule can be accommodated within the binding pore when attached to position 1 of the ligand but not when complexed to streptavidin. The fact that biotin [D-Lys^6]GnRH maintains functionality when complexed to streptavidin while biotin [Gln^1]GnRH does not, suggests that the N- and possibly the C-termini are required for receptor binding. Thus the most likely binding orientation for the ligand is with the N- and C-termini pointing inwards with the residue at position 6 pointing away from the binding site.

Keywords: GnRH, gonadotrophin-releasing hormone; GnRH receptor; Ligand binding; Molecular modelling

1. Introduction

Gonadotrophin-releasing hormone (GnRH) is released from the hypothalamus and acts through receptors present on the pituitary gonadotrophs to regulate the secretion of FSH and LH and, as such, has a critical role in the regulation of reproductive function. The GnRH receptor (GnRH-R) has been cloned from several mammalian species [mouse: (Tsutsumi et al., 1992), rat: (Eidne et al., 1992; Kaiser et al., 1992), cow: (Kakar et al., 1993), human: (Kakar et al., 1992; Chi et al., 1993), sheep: (Brooks et al., 1993; Illing et al., 1993) and pig: (Weesner and Matteri, 1994)] and a non-mammalian species (catfish: Tensen et al., 1997) and has been shown to belong to the large family of 7-transmembrane (7-TM) G-protein coupled receptors (GPCRs). The mammalian GnRH-R has a number of features unique among GPCRs; a complete lack of an intracellular carboxy-terminal tail, substitution of the highly conserved DRY sequence in the second intracellular loop with DRS and the substitution of the conserved Asp in TMII and Asn residues in TMVII with Asn and Asp, respectively.

Due to its pivotal role in controlling reproductive function many analogues of GnRH have been produced (for review see Karten and Rivier, 1986), and GnRH agonists and antagonists have had widespread

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use for the treatment of a number of reproductive disorders. However, to date the precise interactions between the receptor and its ligand which bring about activation and subsequent G-protein coupling remain to be elucidated. This is true for any of the GPCRs. A complete understanding of GnRH ligand/receptor interactions would facilitate the future design of orally active, non-peptide GnRH analogs. To date much information has been gained about the binding characteristics of the ligand by the generation of a large number of GnRH analogs. Although the precise 3-dimensional structures of GnRH and the GnRH-R are unknown some clues about GnRH receptor-ligand interactions have been obtained from site-directed mutagenesis studies and molecular modelling (for review see Sealfon et al., 1997). The initial proposed structures of GnRH came from computational energy minimisation studies which suggested that the bioactive form of GnRH had a β-turn involving the Tyr–Gly–Leu–Arg in positions 5–8 and that there were three low energy conformations of GnRH (Momany, 1976, 1978). One of these, the so-called CC conformer exists with the N- and C-termini in close proximity and this one best explained the observed analog data (Karten and Rivier, 1986). More recent modelling of the GnRH peptide substantiates this hypothesis suggesting a conformation whereby the guanidinium group of Arg8 and the Trp3 side chain are brought into close proximity (Gupta et al., 1993), i.e. the GnRH has a hairpin loop configuration.

The nature of GnRH-R ligand interactions is less clear. It is thought that there is a hydrophilic receptor binding pore within the TM domains into which the ligand binds. Mutagenesis studies have identified a number of amino acid residues within the receptor which appear to be important for ligand binding. Lys121 in TMIII has been shown to be critical for high affinity agonist binding (Zhou et al., 1995) and it was postulated that this residue interacts with His2 of the ligand. Another residue in TMII, Asp98 has also been implicated in high affinity binding of some GnRH agonists (Rodic et al., 1996). Mutating the residue to Glu increased the binding of GnRH but had little effect on the binding of [Trp^2]GnRH, suggesting a possible interaction between Asp98 and His2 of the ligand. As mentioned above one interesting feature of the mammalian GnRH-R is the reciprocal substitution of the highly conserved Asp in TMII and Asn in TMVII with Asn and Asp, respectively. A mouse GnRH-R mutant Asn87Asp has no detectable binding whereas the double Asn87AspAsp318Asn mutant restored high affinity binding (Zhou et al., 1994). It is postulated that in the GnRH-R these two residues interact to form a salt bridge and that they may have an important role in the maintenance of a binding pore. Most recently the side-chain of Arg139 has been implicated as interacting simultaneously with the Asn87 and Asp318 during receptor activation (Ballesteros et al., 1998). Thus there maybe a number of residues within TMII, III and VII that form important interactions with each other or the GnRH ligand.

There are many possible orientations which the GnRH peptide might adopt within the receptor binding pocket. In order to investigate whether the GnRH ligand binds into the putative binding pore with the N- and C-termini or the β-turn of the hairpin loop pointing inwards towards the cell we assessed the ability of two biotinylated GnRH derivatives, biotin [Gln^1]GnRH, biotinylated at position 1 of the ligand and, biotin [D-Lys^6]GnRH biotinylated at position 6 to bind to the GnRH-R and stimulate second messenger function in the form of total inositol phosphate (IP) production. We generated biotin GnRH-streptavidin complexes to assess the effects of the steric hindrance of the streptavidin at these two sites on ligand binding and receptor internalisation.

The pGlu residue at position 1 of GnRH has been shown to be of critical importance for GnRH-induced LH and FSH secretion. Removing the pGlu residue results in a loss of LH secretion from steroid-stimulated, ovariectomised rats (Okada et al., 1973). Substitution of pGlu with Leu, Gly, Pro or Gln gives essentially inactive forms of the peptide in vivo (Fujino et al., 1972; Okada et al., 1973; Sandow et al., 1978). Although the specific role of this residue in binding and/or activation of the receptor has not been fully determined the subsequent universal substitution of pGlu in GnRH antagonists suggests this residue is important in receptor activation. We therefore also investigated the antagonist properties of the biotin [Gln^1]GnRH by assessing its ability to inhibit GnRH-induced total IP production.

2. Materials and methods

2.1. Materials

GnRH, [D-Lys^6]GnRH, des-Gly^{10}, [D-Trp^6]GnRH and [Ac-3,4-dehydro-Pro^1,D-*p*-F-Phe^2, D-Trp^{3,6}]GnRH were obtained from Sigma, Poole, Dorset, UK. Biotin [Gln^1]GnRH was generated by covalently linking a biotin molecule to the amino group of the glutamine residue and was obtained from Peninsula Laboratories (St Helens, Merseyside, UK).

2.2. Biotinylation of [D-Lys^6]-GnRH

[D-Lys^6]GnRH (1.0 mg) and biotin-amidocaproate *N*-hydroxy succinimide ester (0.8 mg) were dissolved in 1.0 ml methanol/dimethyl formamide (10:1, v/v) containing tributylamine (0.01%). The reaction was allowed to proceed at 25°C for 4 h. Resultant products

were then precipitated by the addition of diethyl ether (3.0 ml). After centrifugation, the precipitate was washed with ethyl acetate and re-dissolved in 0.5 ml water containing ammonium acetate (0.1 M) and acetonitrile (6%). The products were separated by HPLC using a 5 μ ODS column, programming the concentration of acetonitrile to 60% in 40 min at a flow rate of 0.8 ml/min. The yield was 77%, based on the absorption at 220 nm.

2.3. Cell culture and receptor expression

HEK-293 cells were used for stable transfection of the rat GnRH-R cDNA. The cells were maintained in DMEM containing 10% heat-inactivated fetal calf serum, 0.3 mg/ml glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (all obtained from Sigma). 3HA tagged rat GnRH-R cDNA was made as previously described (Cook and Eidne, 1997). Wild-type (WT) and 3HA-tagged rat GnRH-R cDNA (3 μg) was stably transfected into monolayer cultures of HEK-293 cells in 60 mm dishes using Transfectam (Promega, Madison, USA). Positive clones were selected using the antibiotic, G418 (Calbiochem, Nottingham, UK) and the presence of the stably expressed GnRH-R assessed by receptor binding assay. The clones used for the study were WT GnRH-R A2s and 3HA-GnRH-R B5s. Membranes prepared from the WT GnRH-R A2 clone bound the GnRH agonist with a K_d of 0.27 nM and a B_{max} of 3.0 pmol/mg protein, while the 3HA-GnRH-R B5 membranes bound with a K_d of 0.20 nM and a B_{max} of 3.1 pmol/mg protein.

2.4. Receptor binding assays

Displacement assays were performed with radiolabelled ^{125}Ides-Gly^{10}, [D-Trp^6]GnRH (100 000 cpm, specific activity 26.4 μCi/μg), 100 μl membranes from HEK-293 A2 cells stably expressing WT GnRH-R, and 250 μl of assay buffer (40 mM tris–HCl, 2 mM $MgCl_2$, pH 7.2). To this was added 50 μl of either GnRH, biotin-[D-Lys^6]GnRH or biotin-[Gln^1]GnRH at final concentrations between 0 and 10^{-5} M in the presence or absence of an excess of streptavidin (Sigma). A ratio of 2.50×10^{-8} moles of streptavidin were added to 10^{-7} moles of GnRH/biotinylated GnRH analog. The samples were incubated at 4°C for 2 h before filtering through a cell harvester (Brander, St. Albans, Herts, UK) onto GFB filters (BDH, Glasgow, UK). In order to assess whether all the biotin [D-Lys^6]GnRH had bound to streptavidin a 20 μl aliquot of 10^{-3} M (equivalent to 20 nmoles) biotin [D-Lys^6]GnRH was passed through a 1 ml tetrameric avidin column (Promega) which bound 33.7 nmoles of biotin/ml. The eluate was freeze-dried and reconstituted to the original starting volume. A 10^{-5} M dose of the eluate was tested in the binding assay and found to be inactive.

2.5. Total IP measurement

The HEK-293 A2 cells were trypsinised, transferred to 24-well plates and labelled with 1.0 μCi/well ^{3}H-myo-inositol in inositol-free DMEM (GIBCO, Paisley, UK), dialysed HIFCS, 0.3 mg/ml glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were incubated for a further 48 h. The transfected cells were washed with 1 mg/ml fatty acid free BSA, 140 mM NaCl, 20 mM HEPES, 4 mM KCl, 8 mM D-glucose, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.2 and incubated for 20 min at 37°C in 10 mM LiCl. The cells were stimulated with varying concentrations ($0–10^{-6}$ M) of GnRH, biotin [D-Lys^6]GnRH or biotin [Gln^1]GnRH for 40 min. In a separate experiment HEK-293 A2 cells stably expressing the receptor were pre-incubated with $0–10^{-6}$ M doses of biotin [Gln^1]GnRH or the antagonist [Ac-3,4-dehydro-Pro^1,D-p-F-Phe^2,D-Trp^{3,6}]-GnRH prior to treatment with the equivalent dose of GnRH. Total IPs were extracted with 2.5% PCA/5 mM EDTA and 1.8 mg/ml of physic acid solution and separated using Ag 1-X8 anion exchange resin (Biorad, Herts, UK). Total IP production is expressed as a percentage of the maximal response to GnRH.

2.6. ELISA

The ELISA assay, for the quantification of 3HA-tagged receptor internalisation, was based on methods previously described (Cook and Eidne, 1997, Daunt et al., 1997). The 3HA GnRH-R B5 cells were plated out at a density of 1.5×10^5 cells/well in 24-well plates. After 24 h, cells were washed once with HEPES-buffered DMEM (Sigma) with 0.1% BSA and then treated with 1 μM GnRH, biotin [D-Lys^6]GnRH or biotin [Gln^1]GnRH peptide in the presence or absence of excess streptavidin for 1 h at 37°C. The cells were washed once with ice-cold PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were washed thrice with Tris buffered saline (TBS) and incubated with blocking buffer (0.1 M $NaHCO_3$, pH 8.6, 1% non-fat milk powder) for 4 h at room temperature. The cells were washed thrice with TBS and incubated with a 1:200 dilution of primary rat anti-HA 3P10 antibody (Boehringer, Mannheim) in blocking buffer overnight at 4°C. Subsequently, cells were washed with TBS (3 ×) and incubated for 1 h at 37°C with a 1:1000 dilution of a horseradish peroxidase-conjugated sheep anti-rat IgG (Sigma). After final washes with TBS (3 ×) the reaction was developed using the 3,3′,5,5′tetramethylbenzidine (TMB) liquid substrate system. The enzymatic reaction was stopped after 30 min at room temperature with 0.5 M H_2SO_4 and 100 μl samples taken for colorimetric measurement at 450 nm.

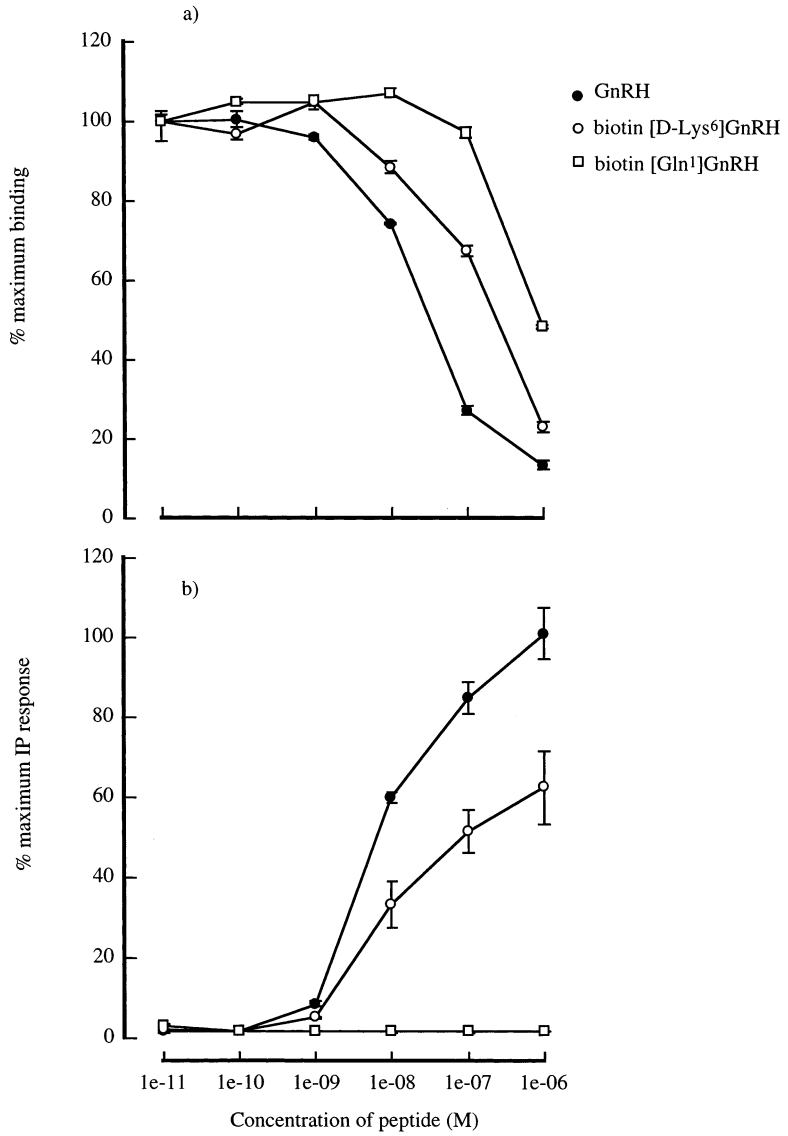

Fig. 1. Displacement binding (a) and total IP production (b) by HEK-293 cells stably expressing the WT GnRH-R in response to $0–10^{-6}$ M doses of GnRH (filled circles), biotin [D-Lys^6]GnRH (open circles) or biotin [Gln^1]GnRH (open squares). The binding ability of each of the ligands was determined by competition binding with ^{125}IdesGly^{10},[D-Trp^6]-GnRH. Data are representative of three experiments performed in triplicate.

2.7. Statistical analysis

Differences between the ability of GnRH and the biotinylated GnRH analogs to bind to the receptor, stimulate second messenger function and cause receptor internalisation were analysed using *t*-tests. A value of $P < 0.05$ was considered significant.

3. Results

3.1. GnRH and biotinylated GnRH peptides: receptor binding and total IP stimulation

In a displacement binding assay with ^{125}Ides-Gly^{10}, [D-Trp^6]GnRH, GnRH binding to the GnRH-R was shown to have an IC_{50} of 32.9 ± 9.6 nM (Fig. 1a, Table 1). GnRH stimulated total IP production with an ED_{50} of 25.3 ± 15.9 nM (Fig. 1b, Table 1). Both the biotin [D-Lys^6]GnRH and biotin [Gln^1]GnRH receptor binding had significantly increased IC_{50} values (104.3 ± 16.6 and 271 ± 41.2 nM, respectively, Fig. 1a, Table 1). However, only the biotin [D-Lys^6]GnRH was able to stimulate total IP production with an ED_{50} value of 256 ± 99.3 nM (Fig. 1b, Table 1).

3.2. Antagonist properties of biotin [Gln^1]GnRH

In order to investigate the possible antagonist properties of biotin [Gln^1]GnRH, the GnRH-induced IP production by cells stably expressing the GnRH-R was determined. Cells were pre-incubated with either

medium (control), a GnRH antagonist [Ac-3,4-dehydro-Pro[1],D-*p*-F-Phe[2],D-Trp[3,6]]-GnRH or biotin [Gln[1]]GnRH before being treated with GnRH (10^{-8}–10^{-6} M doses). Pre-incubation with the 10^{-8}–10^{-6} M doses of antagonist [Ac-3,4-dehydro-Pro[1],D-*p*-F-Phe[2],D-Trp[3,6]]-GnRH completely abolished the GnRH-induced total IP production (Fig. 2). Pre-incubation with the 10^{-8}–10^{-6} M doses of biotin [Gln[1]]GnRH significantly reduced ($P < 0.05$) the GnRH-induced total IP response. These data suggest that biotin [Gln[1]]GnRH acts as a weak antagonist.

3.3. GnRH and biotinylated GnRH peptides: receptor binding in the presence of excess streptavidin

GnRH bound to the receptor with an IC_{50} of 32.9 ± 9.6 nM and this affinity for the receptor was not significantly altered by pre-incubation with an excess of streptavidin, $IC_{50} = 40.3 \pm 7.1$ nM (Fig. 3a, Table 1). The biotin [D-Lys[6]]GnRH–streptavidin complex was able to bind to the receptor although the IC_{50} value was significantly increased (1230 ± 1186 nM, $P < 0.05$) compared to the biotin [D-Lys[6]]-GnRH alone (Fig. 3b, Table 1). In the presence of excess streptavidin the biotin [Gln[1]]GnRH was unable to bind to the receptor (Fig. 3c, Table 1).

Table 1
Functional characteristics of the GnRH peptides

Peptide	Displacement binding (IC_{50} nM)	GnRH-induced IP production (ED_{50} nM)
GnRH	32.9 ± 9.6	25.3 ± 15.9
Biotin [D-Lys[6]]GnRH	104 ± 16.6*	256 ± 99*
Biotin [Gln[1]]GnRH	271 ± 41.4*	Basal
GnRH + streptavidin	40.3 ± 7.1	Nd
Biotin [D-Lys[6]]GnRH + streptavidin	1230 ± 1186*	Nd
Biotin [Gln[1]]GnRH + streptavidin	No binding	Nd

The IC_{50} values for binding are calculated from competition displacement assays and the ED_{50} values for IP production from dose dependent GnRH/biotinylated GnRH analog stimulated total IP assays. For the GnRH peptide + streptavidin displacement assays 2.50×10^{-8} moles of streptavidin were added to 10^{-7} moles of GnRH/biotinylated GnRH analog. Values represent the mean ± SEM from at least $n = 3$ independent experiments.
* Represents a significant change ($P < 0.05$) in IC_{50}/ED_{50} value compared to GnRH.
Nd = not determined.

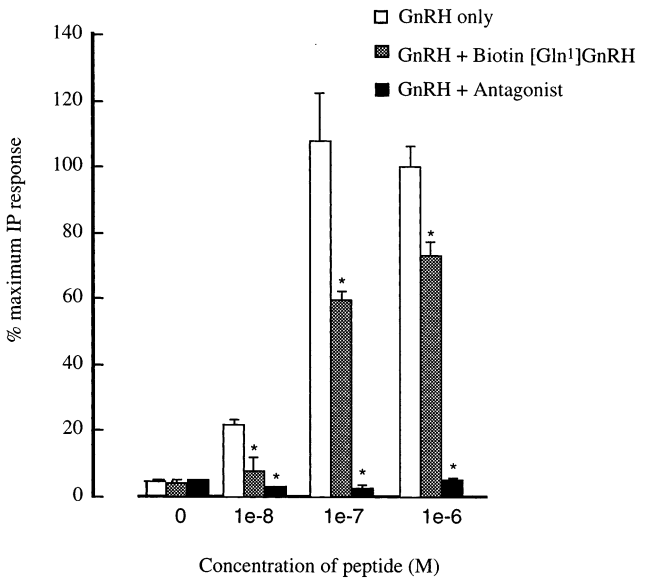

Fig. 2. Total IP production by HEK-293 cells stably expressing the GnRH-R in response to 10^{-8}–10^{-6} M doses of GnRH only (open bars), GnRH and biotin [Gln[1]]GnRH (shaded bars) or GnRH and the GnRH antagonist, Ac-3,4-dehydro-Pro[1],D-*p*-F-Phe[2],D-Trp[3,6]]-GnRH (filled bars). Cells were pre-treated with the 10^{-8}–10^{-6} M doses of biotin [Gln[1]]GnRH or GnRH antagonist for 20 min prior to the addition of GnRH. Data are representative of three experiments performed in triplicate. Significant differences between the total IP production in cells treated with GnRH only or pre-treated with biotin [Gln[1]]GnRH or GnRH antagonist, are shown, *$P <$ 0.05.

3.4. ELISA measurement of receptor internalisation in response to GnRH and biotinylated GnRH peptides using 3HA-tagged GnRH-R

The ability of the biotinylated GnRH analogs to bind and activate the receptor in the presence of excess SA was confirmed using an ELISA to measure receptor internalisation (Fig. 4). Following a one hour treatment with GnRH $72.9 \pm 3.8\%$ of the receptors remained on the surface of the HEK-293 cells stably expressing the HA-tagged GnRH-R compared to untreated control, and this reduction in the levels of receptor at the cell surface was not affected by the presence of excess streptavidin. Treatment with biotin [D-Lys[6]]GnRH also stimulated receptor internalisation with a reduction in the cell surface receptor levels to $75.1 \pm 2.4\%$ of the untreated control (Fig. 4). In the presence of streptavidin this level of internalisation was maintained with a reduction in the cell surface receptor number to $72.7 \pm 6.5\%$. Biotin [Gln[1]]GnRH acts as an antagonist and therefore does not stimulate second messenger function or receptor internalisation. Streptavidin only treatment of the cells had no significant effect on receptor internalisation when compared to untreated control.

4. Discussion

To date the definitive structure for both the GnRH-R and the GnRH ligand remain to be resolved and the nature of GnRH-R-ligand interactions is unclear. A model of the GnRH ligand suggests a β-turn involving the Tyr–Gly–Leu–Arg in positions 5–8 (Momany, 1976, 1978). Further analysis suggests the GnRH ligand forms a hairpin loop structure with the N- and C-termini in close proximity (Gupta et al., 1993). Models of the GnRH-R are more difficult to generate due to the lack of structural data available for any GPCR. Co-ordinates of the trans-membrane domains for the GnRH-R have been estimated (Baldwin, 1993; Baldwin et al., 1997) from low resolution projection maps of rhodopsin (Schertler et al., 1993; Schertler and Hargrave, 1995) and bacteriorhodopsin (Grigorieff et al., 1996). This together with information obtained from site-directed mutagenesis studies has allowed modelling of the putative binding pore. It is postulated that the GnRH ligand binds to the receptor between trans-membrane domains II, III and VII (Sealfon et al., 1997), but the binding orientation of the ligand to this hydrophilic receptor pore is as yet unknown.

There are several possible orientations for the ligand to bind to the putative receptor binding pore and of these we investigated whether the ligand binds with the N- and C-termini or the β-II turn pointing towards the cell. We assessed the ability of two biotinylated GnRH analogs to both bind to the receptor, stimulate second messenger function and cause receptor internalisation in the presence and absence of streptavidin. The mammalian form of GnRH displaced radiolabelled GnRH agonist and stimulates total IP with IC_{50}/ED_{50} values in the 10 nM range. Biotin [D-Lys^6]GnRH, biotinylated at

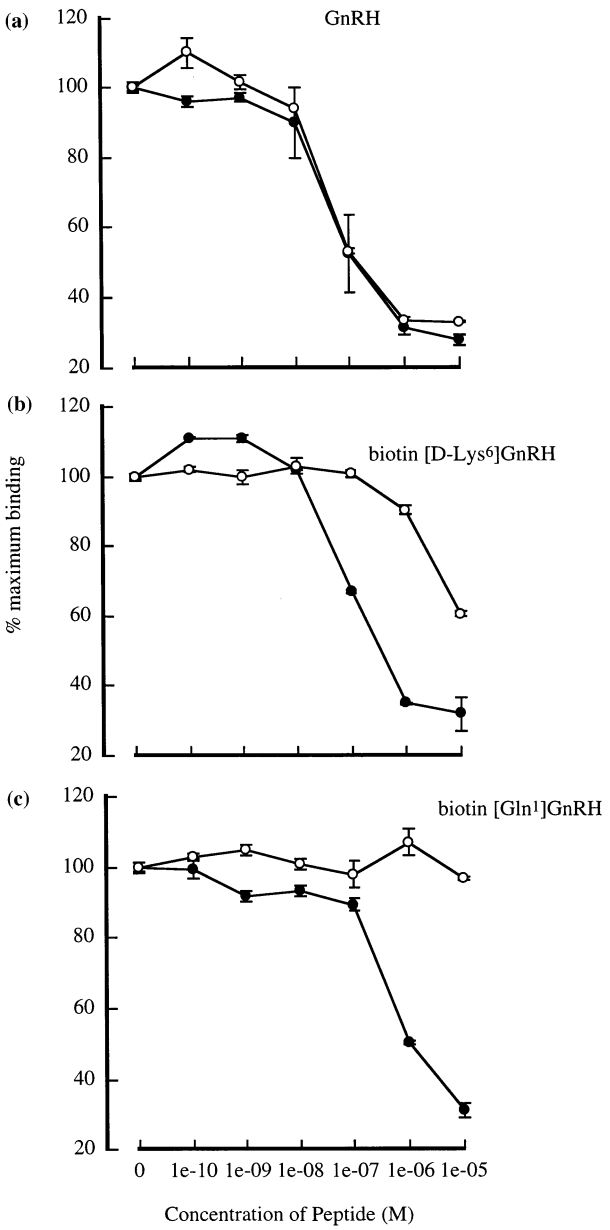

Fig. 3. Displacement binding by membranes prepared from HEK-293 cells stably expressing the GnRH-R in response to $0–10^{-5}$ M doses of (a) GnRH, (b) biotin [D-Lys^6]GnRH and (c) biotin [Gln^1]GnRH in the absence (closed circles) and presence (open circles) of excess streptavidin. GnRH and the biotinylated ligands were preincubated with streptavidin for 20 min at room temperature prior to addition to the binding assay. The binding ability of each of the ligands was determined by competition binding with ^{125}Ides-Gly^{10},[D-Trp^6]-GnRH.

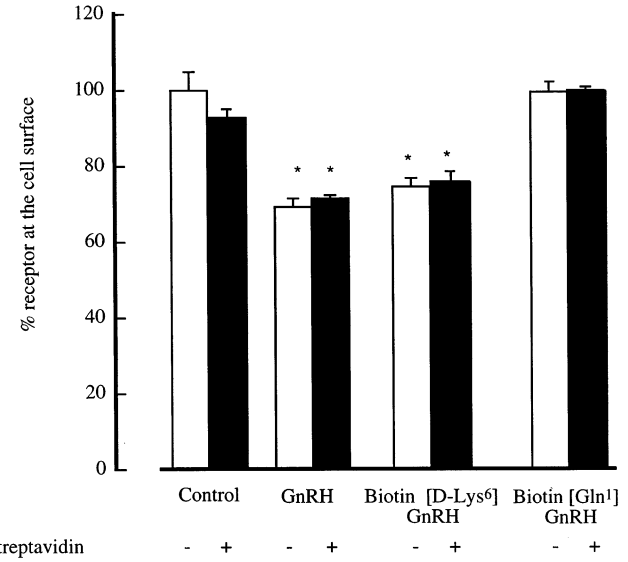

Fig. 4. Elisa measurement of the cell surface receptor number of HEK-293 cells stably expressing HA tagged GnRH-R after treatment with medium only (control), streptavidin only (control), GnRH + / − streptavidin, biotin [D-Lys^6]GnRH + / − streptavidin or biotin [Gln^1]GnRH + / − streptavidin. GnRH and the biotinylated ligands were preincubated with streptavidin for 20 min at room temperature prior to addition to the cells. A 10^{-6} M concentration of GnRH or the biotinylated ligands was used for each experimental condition. Data are representative of three experiments performed in triplicate. Significant differences between the treatments and the untreated cells are shown, * = $P < 0.05$.

position 6 binds to the receptor and stimulates total IP with significantly increased IC_{50}/ED_{50} compared to GnRH. The biotin [Gln[1]]GnRH, biotinylated at position 1, also binds to the receptor but does not stimulate second messenger function.

The possible antagonist properties of the biotin [Gln[1]]GnRH were further investigated by performing a total IP assay with cells that had been pre-incubated with either medium alone, a GnRH antagonist [Ac-3,4-dehydro-Pro[1],D-*p*-F-Phe[2],D-Trp[3,6]]-GnRH or biotin [Gln[1]]GnRH prior to treatment with a range of concentrations of GnRH. Pre-incubation of the GnRH-R with the GnRH antagonist abolished GnRH-induced total IP production while pre-incubation with biotin [Gln[1]]GnRH significantly reduced the GnRH-induced response. These data demonstrate that biotin [Gln[1]]GnRH acts as an antagonist, albeit a weak one and would suggest that the pGlu amino acid at position 1 is important for receptor activation. Previous studies have shown that administration of GnRH analogs with substitutions in this position to ovariectomised rats, fails to elicite a GnRH-induced LH response (Fujino et al., 1972; Okada et al., 1973; Sandow et al., 1978). However His2 and Trp3 of the ligand are the only residues for which good evidence exists showing a direct involvement in receptor activation (Yanihara et al., 1973; Coy et al., 1975). It is also possible that the biotin-Gln residue at position 1 distorts the ligand binding pocket to such an extent as to interfere with the interactions between His2 and Trp3 of the ligand and residues within the receptor which result in receptor activation.

It has been suggested that GnRH antagonists may have a different binding site within the GnRH-R to GnRH agonists. Mutating Lys121 of the receptor to Glu resulted in a loss of agonist binding, however statistical analysis of the data suggested that the Lys121Glu mutant would have retained antagonist binding (Zhou et al., 1995). Another study used photoaffinity-labelled agonist and antagonist to show different binding sites for the two peptides (Janovick et al., 1993). Therefore, it may be that the biotin [Gln[1]]GnRH binds to a different site within the receptor binding pore to the GnRH and biotin [D-Lys[6]]-GnRH.

The ability of the biotinylated GnRH analogs to bind to the receptor in the presence of streptavidin was also assessed. While the presence of excess streptavidin had no effect on the binding ability of GnRH, the ability of biotin [D-Lys[6]]GnRH to bind to the receptor was significantly reduced. This is not surprising, since each streptavidin molecule binds four biotin molecules, a decrease in the amount of peptide available to bind into the receptor would be expected. To exclude the possibility that binding of the biotin [D-Lys[6]]GnRH streptavidin complex was due to contamination by either unbiotinylated [D-Lys[6]]GnRH or [D-Lys[6]]GnRH coupled to altered or damaged biotin an aliquot was passed over a tetrameric avidin column. The eluate was tested for its ability to bind and shown to be inactive. In contrast, in the presence of excess streptavidin, the ability of the biotin [Gln[1]]GnRH to bind to the receptor was abolished. This data was confirmed using an ELISA to measure receptor internalisation in response to GnRH, biotin [D-Lys[6]]GnRH and biotin [Gln[1]]GnRH in the absence and presence of streptavidin. The receptor internalisation in response to GnRH and biotin [D-Lys[6]]GnRH was unaffected by the presence of streptavidin. As the biotin [Gln[1]]GnRH acts as an antagonist and is unable to stimulate second messenger function it does not cause receptor internalisation. In order to complete all the controls for the experiment it would have been necessary to pre-incubate an unmodified form of [Gln[1]]GnRH with streptavidin to assess the affects of this on receptor binding. However this compound is extremely unstable, with the Gln at position 1 cyclizing to pGlu, the residue found in the native form of mammalian GnRH (personal communication, Penninsula Laboratories).

The 60 kDa streptavidin molecule is larger than the entire receptor and it is unlikely that it would be able to enter the receptor pore; in this case attaching it to GnRH at the N terminus produced a totally inactive derivative, whereas the biotin [D-Lys[6]]GnRH proved to be capable of binding and caused receptor internalisation. These data are highly suggestive of the GnRH binding to the receptor with the N- and possibly the C-termini pointing inwards towards the cell. This is also supported by the fact that the radiolabelled forms of GnRH agonists have a large iodine group attached to the Tyr at position 5 of the GnRH molecule and are also able to bind to the receptor. However, we have not investigated the effects of the large streptavidin, attached to the C-terminus of the ligand, on receptor binding. It is possible that attachment at this site would allow ligand binding, however there is much data to suggest that the C-terminus of GnRH is integral in receptor binding (Sealfon et al., 1997). If we assume that GnRH binds with the N- and C-termini pointing towards the cell, we are still left with two further possible binding orientations. The indole of Trp3 may face towards either TM helices II and III or TM helix VII. Further studies are required to elucidate the precise binding orientation of the ligand within the receptor pore.

A number of amino acid residues within the extracellular loops of the receptor have been identified as being important for ligand binding (Flanagan et al., 1994; Davidson et al., 1996). To date, no structural data is available for the extracellular regions of any 7-TM receptor making the modelling of these areas very difficult. However it is likely that there are a host of

interactions between the ligand and receptor prior to the ligand reaching its final resting place in the putative binding pore between TM II, III and VII with the N- and C-termini pointing towards the cell.

In conclusion we present data to suggest that the GnRH ligand binds with the N- and C-termini pointing inwards towards the cell and the βII turn projecting away from the binding pore. These data also further support molecular models of the GnRH-R which postulate a binding site between TM II, III and VII.

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