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Hexapeptide and Cyclic Pentapeptide Endothelin Antagonists Directly Activate Pituitary Gonadotropin-Releasing Hormone Receptors

DROR YAHALOM, YITZHAK KOCH, NURIT BEN-AROYA, and MATI FRIDKIN

Departments of Neurobiology (D.Y., Y.K., N.B.-A.) and Organic Chemistry (D.Y., M.F.), Weizmann Institute of Science, Rehovot, Israel

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ABSTRACT

In the course of our studies toward the development of novel analogs of the decapeptide gonadotropin releasing hormone (GnRH), we have examined a hexapeptide that is an antagonist of endothelin (ET). It was found that this peptide, Ac-D-Trp-Leu-Asp-Ile-Ile-Trp (peptide 1), binds specifically to the pituitary GnRH receptor. Moreover, peptide 1 exhibits a GnRH agonistic activity (i.e., it induces luteinizing hormone release from rat pituitary). This activity is mediated directly by the GnRH receptor and is suppressed by a GnRH antagonist. Removal of the acetyl group of peptide 1 results in a hexapeptide (peptide 2) with binding properties similar to those of GnRH but with a diminished affinity toward the ET receptor. Several other ET antagonists were screened for a potential interaction with the GnRH receptor. Two of these, the hexapeptide PD145065 and the cyclic pentapeptide BQ-123, expressed GnRH agonistic activity at micromolar concentrations in vitro. BQ-123, previously approved for trials on humans as an ET antagonist, is demonstrated to act in vivo as a GnRH agonist, in a dose that was demonstrated previously as the minimal required dose for significant ET antagonism. The GnRH agonistic activity of ET antagonists may therefore result in interference with the physiological control of the reproductive system. Such effects may be most severe when ET antagonists are used chronically. Thus, the major practical message of this study is the need to circumvent the potential side effects of ET antagonist-based drugs.

Gonadotropin-releasing hormone (GnRH; pyroglutamic acid-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is a neurohormone that regulates the reproductive system by controlling the secretion of the gonadotropic hormones, luteinizing hormone (LH) and follicle-stimulating hormone, from the anterior pituitary (Amoss et al, 1971; Matsuo et al., 1971). The neurohormone is physiologically released in a pulsatile pattern, whereas its continuous administration results in down-regulation and receptor desensitization, leading to an inhibition of pituitary gonadotropin secretion (Belchetz et al., 1978; Gallo, 1980). Therefore, synthetic GnRH analogs may be used for contraception and for the therapy of various hormone-dependent diseases including prostate and breast cancer (Emons et al., 1994).

The endothelins (ETs) are a family of bicyclic 21-amino acid peptides. ET-1 is the most potent vasoconstrictor known, being about 10-fold more potent than angiotensin II (Cody and Doherty, 1995). Other peptides of this family (i.e., ET-2 and ET-3) are also powerful vasoconstrictors. Two ET receptors were cloned and characterized: ET_A receptors are more specific for ET-1 and ET-2 than for ET-3, whereas ET_B receptors are nonselective (Arai et al, 1990; Sakurai et al., 1990). There are indications that at least two additional receptor subtypes exist (Sokolovsky, 1994). ET-1 was shown to stimulate LH release independently of the GnRH receptor, probably via pituitary ET_A receptors (Stojilkovic et al., 1990, 1992).

The potential pharmacological importance of ETs, especially in the cardiovascular system, has led to accelerated development of synthetic analogs, with emphasis on potent and selective antagonists (Cody and Doherty, 1995). One of the approaches to develop such analogs is based on alteration or modification of the carboxyl-terminal hydrophobic hexapeptide, ET[16–21], which is identical in all ETs. Structure-activity relationship studies of this fragment using systematic replacements of amino acid residues by D-amino acids and unnatural amino acids has led to the development of very potent antagonists (Doherty et al., 1993). Parallel stud-

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ABBREVIATIONS: GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; ET, endothelin; peptide 1, Ac-D-Trp-Leu-Asp-Ile-Ile-Trp; Antide, Ac-β-[2-Naphtyl]-D-Ala-D-*p*-Chloro-Phe-β-[2-Pyridyl]-D-Ala-Ser-N^{ϵ}-[Nicotinoyl]-Lys-N^{ϵ}-[Nicotinoyl]-D-Lys-Leu-N^{ϵ}-[Isopropyl]-Lys-Pro-D-Ala-NH_2; DMSO, dimethyl sulfoxide; peptide 2, D-Trp-Leu-Asp-Ile-Ile-Trp; RIA, radioimmunoassay.

ies based on the isolation of natural ET antagonists led to the design of very potent cyclic pentapeptide and linear tripeptide ET antagonists (Ihara et al., 1992; Ishikawa et al., 1992, 1994).

In contrast to the successful development of reduced-size ET antagonists, all of the GnRH antagonists used in current clinical trials are rather equivalent in size to GnRH itself. Potent GnRH agonists, some of which are already used for clinical purposes, are either decapeptides or nonapeptides (Filicori, 1994). Although thousands of GnRH analogs were synthesized, only very few reports on further reduced-size GnRH analogs were published (Sandow and Konig, 1979; Haviv et al., 1989; Janecka et al., 1995) because deletions of either amino- or carboxyl-terminal amino acids cause a dramatic loss of bioactivity (Karten and Rivier, 1986). A representative example of these small ligands is a hexapeptide corresponding to amino acid residues 4–9 of the potent GnRH analog buserelin. The in vivo potency of this fragment for LH release in the rat is 3.8% of the respective potency of GnRH, and only 0.2% of the respective potency of buserelin (Sandow and Konig, 1979).

In the course of our studies toward the development of novel GnRH analogs, we tested a hexapeptide ET antagonist derived from ET[16–21] that has no apparent sequence similarity to GnRH. In this article, we show that this peptide, Ac-D-Trp-Leu-Asp-Ile-Ile-Trp (peptide 1) (Doherty et al., 1993) is a GnRH agonist (i.e., it binds specifically to pituitary GnRH receptors and induces LH release). A GnRH antagonist can block its activity. We also present preliminary structure-activity relationship studies and address the issue of the potential interaction of other ET antagonists with the GnRH receptor.

Experimental Procedures

Materials. Unless otherwise stated, all chemicals and reagents were of analytical grade. Trifluoroacetic acid for HPLC was obtained from Merck (Darmstadt, Germany). 9-Fluorenylmethoxycarbonyl-protected amino acid derivatives, Wang resin, and Rink-amide resin were purchased from Novabiochem (Laufelfingen, Switzerland). [D-Trp^6]GnRH was purchased from Bachem (Torrance, CA). Ac-β-[2-Naphtyl]-D-Ala-D-*p*-Chloro-Phe-β-[3-Pyridyl]-D-Ala-Ser-N$^\epsilon$-[Nicotinoyl]-Lys-N$^\epsilon$-[Nicotinoyl]-D-Lys-Leu-N$^\epsilon$-[Isopropyl]-Lys-Pro-D-Ala-NH_2 (Antide), PD145065, and ET-1 were acquired from Sigma (St. Louis, MO). BQ-123 (for experiments described in Table 3 and Figs. 2 and 3A), BQ-788, and FR139317 were purchased from Neosystem (Strasbourg, France). BQ-123 (for the experiment described in Fig. 3B) was obtained from Bachem AG (Dubendorf, Switzerland), and BQ-610 was obtained from Peninsula Laboratories (Belmont, CA).

Peptide Synthesis. All peptides, other than the above, were prepared in our laboratory by solid-phase peptide synthesis, with an AMS-422 multiple peptide synthesizer (Abimed Analysentechnik GmbH, Langenfeld, Germany) using 9-fluorenylmethoxycarbonyl chemistry (Atherton and Sheppard, 1989) following the protocols of the company. The crude peptides were subjected to semipreparative HPLC purification, performed on a Waters system composed of two model 510 pumps, a model 680 automated gradient controller, and a model 441 absorbance detector (Waters, Milford, MA). The column effluents were monitored by UV absorbance at 214/254 nm. HPLC prepacked columns (Merck, Darmstadt, Germany) were LichroCART 250–10 mm containing Lichrosorb RP-18 (7 μm) for semipreparative purifications and Lichrospher 100 RP-18, 250–4 mm (5 μm) for analytical separations. Separations were achieved using gradients of acetonitrile in water containing 0.1% trifluoroacetic acid. The homogeneity of the resulting peptides was tested by analytical HPLC to be higher than 97%. Solutions containing purified peptides were lyophilized. Samples of each of the peptides were hydrolyzed (6 N HCl, 110°C, 22 h, in vacuum) and analyzed with a Dionex automatic amino acid analyzer. These results were also used for quantification of the peptide content in each preparation. The peptides were also analyzed by an LCQ mass spectrometry system (Finnigan, Bremen, Germany) using a nanospray ionization technique. The latter two analyses further confirmed the composition and purity of the products. Pure peptides were dissolved in water [or in dimethyl sulfoxide (DMSO) in the case of the hydrophobic peptide 1 and its nonacetylated form D-Trp-Leu-Asp-Ile-Ile-Trp (peptide 2)] to obtain 1 mM concentration, and aliquots were kept frozen (−20°C). The DMSO content in the preparations for bioassays of peptide 2 and peptide 1 (1% or lower) was tested and found to have no effect on GnRH binding or LH release.

Animal Studies. Wistar-derived rats were obtained from the institution's Research Animal Resource Center. Experiments were carried out in compliance with the regulations of the Weizmann Institute of Science.

Binding to the Pituitary GnRH Receptor. [D-Lys^6]GnRH (synthesized in our laboratory) was iodinated by the chloramine T method and ^{125}I-[D-Lys^6]GnRH was purified (1700 μCi/nmol) by analytic HPLC system as described above. The binding assay was conducted as described previously (Yahalom et al., 1999) In brief, pituitary membranes (25 μg of protein/tube, prepared from Wistar-derived proestrous rats) were incubated for 90 min at 4°C with 50,000 cpm (23.5 pM) ^{125}I-[D-Lys^6]GnRH, alone or in the presence of various concentrations of the unlabeled peptides, in a total volume of 0.5 ml of the assay buffer (10 mM Tris · HCl containing 0.1% BSA). The reaction was terminated by rapid filtration through Whatman GF/C filters. The filters were washed three times with cold assay buffer and counted in an Auto-Gamma Counting System (Packard, Meriden, CT). The experiments were performed in triplicate. Nonspecific binding was defined as the residual binding in the presence of excess of [D-Lys^6]GnRH (1 μM). Specific binding was calculated by subtracting the nonspecific binding from the maximal binding, determined in the absence of any competing peptide. IC_{50} values were calculated using the curve-fitting software program Enzfitter (Elsevier Biosoft, Cambridge, UK).

Binding of Peptides to ET Receptors. ^{125}I-ET-1 was prepared as reported previously (Girsh et al., 1996) and purified (1800 μCi/nmol) by analytic HPLC system as described above. Preparation of membranes from both the pituitary gland and brain tissues, as well as the binding assay, were performed in a procedure that is similar to that described for the GnRH receptor binding assay, except for several differences. Random cycling rats were used instead of proestrous rats; each tube contained 50,000 cpm (25 pM) ^{125}I-ET-1 and either membranes from brain (400 μg of protein/tube) or from pituitary (50 μg of protein/tube). The samples were incubated at 37°C for 120 min. The nonspecific binding was determined in the presence of 1 μM ET-1.

LH Release from Cultured, Dispersed, Pituitary Cells. Cells from 21-day-old Wistar-derived female rats were dispersed as described previously (Liscovitch et al., 1984) and incubated in 96-well plates (50,000 cells/well) at 37°C in M-199 medium containing 5% horse serum. After 48 h the cells were washed with M-199 medium containing 0.1% BSA and incubated for 4 h at 37°C with M-199/0.1% BSA (0.25 ml) containing the desired concentrations of the various peptides (four wells per experimental group). The incubation was terminated by removing the medium and diluting it by an equal volume of 1% BSA in PBS. Three aliquots from each sample were analyzed for LH concentration by radioimmunoassay (RIA) (Daane and Parlow, 1971) using the kit kindly supplied by the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD) Rat Pituitary Program. Results are expressed in terms of the RP-3 reference preparation.

In Vivo LH Release. PBS (0.5 ml of 0.1 M) containing the desired concentration of BQ-123 was i.p. injected to rats (220 ± 10 g body

weight; six per group) during the morning of proestrous. The control group was injected with 0.5 ml of 0.1 M PBS containing the equivalent amount of DMSO. Blood samples were withdrawn by cardiac puncture under light ether anesthesia at the indicated time after drug administration. The serum samples were assayed for LH content as described above.

Statistical Analysis. Results are expressed as the mean ± S.E. Comparisons were made using one-way ANOVA (Instat 2.01; GraphPad Software, San Diego, CA). P values $< .05$ were taken as significant.

Results

Peptide 1, derived from the sequence of the carboxyl-terminal domain of ET, binds specifically to pituitary GnRH receptors with a moderate affinity, whereas ET-1 does not bind to the GnRH receptor (Table 1). GnRH does not compete for the ET receptors (Table 1); nor do other GnRH analogs such as [D-Trp^6]GnRH, [D-Lys^6]GnRH, and Antide (a GnRH antagonist), which did not interact with the ET receptors (data not shown). Peptide 2 binds to the GnRH receptor (Table 1), although its affinity is somewhat lower than the affinity of peptide 1. As expected from previous studies (Doherty et al., 1993), the omission of the acetyl group in peptide 2 results, however, in a diminished ET receptor-binding capacity (Table 1). The binding data shown in Table 1 are the results of binding assays with pituitary gland membranes rich in ET_A receptors. Similar results were obtained using brain membranes (data not shown), where ET_B is predominant (Williams et al., 1991; Kanyicska and Freeman, 1993).

Peptides 1 and 2 are both capable of inducing LH release from rat pituitaries, and their potencies match their binding affinities (Fig. 1). The inability of peptide 2 to compete for ET receptor binding (Table 1) provides evidence that the effect of peptide 2 on LH release is not mediated via the ET receptor. To further establish the role of GnRH receptors as mediators of the activity of peptides 1 and 2 on LH release, we have examined the possibility that the activity of these peptides will be inhibited by the presence of a GnRH antagonist (Fig. 2). Indeed, the LH-releasing activity of peptides 1 and 2, as well as that of GnRH, were diminished in the presence of the GnRH antagonist (Fig. 2). Taken together, these observations strongly suggest that the activity of peptide 1 is mediated directly by GnRH receptors. In contrast, as demonstrated earlier (Stojilkovic et al., 1990), we have also found that the activity of ET-1 was not affected by the addition of a GnRH antagonist (data not shown); i.e., the LH secretory response to ET-1 is independent of the GnRH receptor.

Structure-activity studies were performed to gain better insight into the recognition of the GnRH receptor by peptide 1. We have examined the possibility that receptor recognition is based on a structural similarity between peptide 1 and the carboxyl-terminal domain of the potent GnRH agonist [D-Trp^6]GnRH (Table 2). To test this hypothesis, we have constructed a chimera in which the carboxyl terminus of [D-Trp^6]GnRH was replaced by the sequence of peptide 2; the D-Trp-Leu dipeptide found in both peptides connects the two parts of the molecule (chimera 1; see Table 2). The interaction of chimera 1 with the GnRH receptor is of low affinity (Table 2). Modification of this chimera by using motifs found in the original carboxyl-terminal domain of GnRH, such as the carboxyl-terminal amide (chimera 2) or the Arg residue (chimera 3), resulted in increased GnRH receptor recognition (Table 2). On the other hand, the acetylated carboxyl-terminal hexapeptide of [D-Trp^6]GnRH has GnRH receptor affinity that is similar to that of peptide 1 (Table 2). Taken together, these results suggest that the sequence of peptide 1 may resemble that of [D-Trp^6]GnRH, but it is not an appropriate substitution for the carboxyl-terminal domain of decapeptide GnRH analogs.

A preliminary search for pharmacophores in the peptide 1 sequence showed that the deletion of either of the D-Trp residues in peptide 1, or the replacement of Asp by Asn, resulted in diminished receptor recognition of the respective peptides (data not shown). In addition, replacement of the D-Trp residue by L-His, which is the original residue in the ET carboxyl terminus sequence, also resulted in loss of affinity for the GnRH receptor (Table 1).

We next tested various commercially available ET antagonists for their potential interactions with the GnRH receptor. Peptides tested were: 1) the nonselective ET antagonist PD145065, a hexapeptide closely related to peptide 1 (Cody and Doherty, 1995); 2) BQ-123, an ET_A-selective cyclic pentapeptide antagonist (Ihara et al., 1992); 3) BQ-788, an ET_B-selective tripeptide antagonist (Shacoori et al., 1995); 4) FR139317 (Sogabe et al., 1993); and 5) BQ-610 (Ishikawa et al., 1992), ET_A-selective tripeptide antagonists. Binding studies (Table 3) and functional studies (Fig. 2) both demonstrate specific interaction of PD145065 and BQ-123 with the GnRH receptor. Antide, a specific GnRH antagonist (Fig. 2), inhibits the LH-releasing activity of these peptides. The other ET antagonists that were studied, the tripeptides BQ-

TABLE 1

Binding affinities of peptides to the GnRH and endothelin receptors

K_i is the inhibition constant for the displacement of specific binding of ^{125}I-[D-Lys^6]GnRH (K_d = 177 pM) or of ^{125}I-ET-1 (K_d = 2 nM) bound to rat pituitary membranes. Values are based on displacement curves obtained by incubating pituitary membranes for 90 min at 4°C with the respective tracer and increasing concentrations of the unlabeled peptides, as described in *Experimental Procedures*. Nonspecific binding was determined in the presence of 1 μM of GnRH or of ET-1 and subtracted from the total binding for the calculation of specific binding. Results are mean ± S.E. of two experiments carried out in triplicate.

Peptide	Sequence	K_i	
		GnRH Receptor	ET Receptor
		μM	
ET-1		NB	0.002
ET[16–21]	His-Leu-Asp-Ile-Ile-Trp-OH	NB	NB
Peptide 1	Ac-D-Trp-Leu-Asp-Ile-Ile-Trp-OH	1.9 ± 0.5	0.12 ± 0.07
Peptide 2	D-Trp-Leu-Asp-Ile-Ile-Trp-OH	8 ± 1	55 ± 15
GnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	0.002	NB

NB, no significant binding at 10 μM; pGlu, pyroglutamic acid.

610, BQ-788, and FR139317, had a very low GnRH receptor binding affinity (Table 3) and consequently did not stimulate LH release when incubated with dispersed pituitary cells at 1 μM (data not shown).

BQ-123, the most potent ET antagonist in inducing LH release from pituitary cells (Fig. 2), was tested for its in vivo LH-releasing activity in proestrous rats after i.p. administration (Fig. 3). At a dose of 100 μg/kg, which is regarded as the minimal dose required to induce a significant ET receptor-mediated antihypertensive effect in rats (Mihara et al., 1994), BQ-123 induces a highly significant ($P < .001$) increase in serum LH levels (Fig. 3A). A dose dependence study (Fig. 3B) demonstrated that 100 μg/kg of BQ-123 induced maximal LH release, which reached plateau at a higher dose. We have also found that this response is equivalent to the effect of 0.5 μg/kg of GnRH, and a further increase of GnRH doses (5 and 50 μg/kg) induced higher rates of LH release (data not shown).

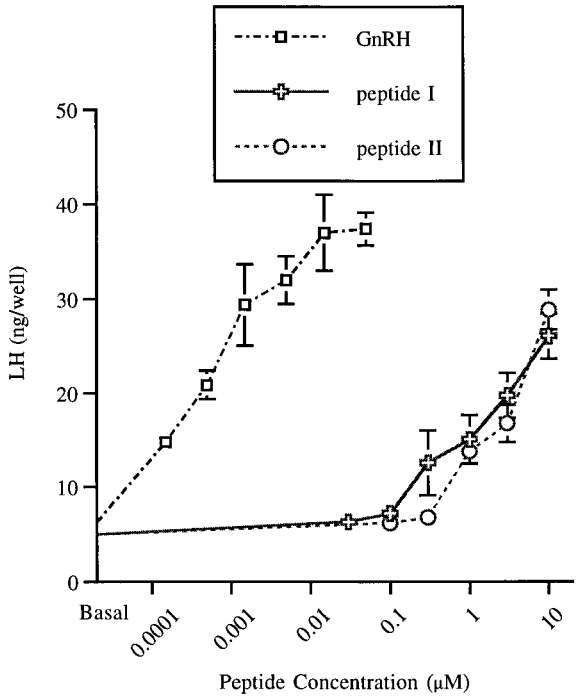

Fig. 1. Dose-response curves for the induction of LH release from dispersed rat pituitary cells by GnRH and peptides 1 and 2. Cells were preincubated for 48 h after dispersion and incubated for 4 h at 37°C with the examined peptides, followed by determination of LH in the medium using RIA. Results are the mean ± S.E. of LH concentration in two experiments (four wells per experimental group). The LH concentration in each well was determined using triplicates.

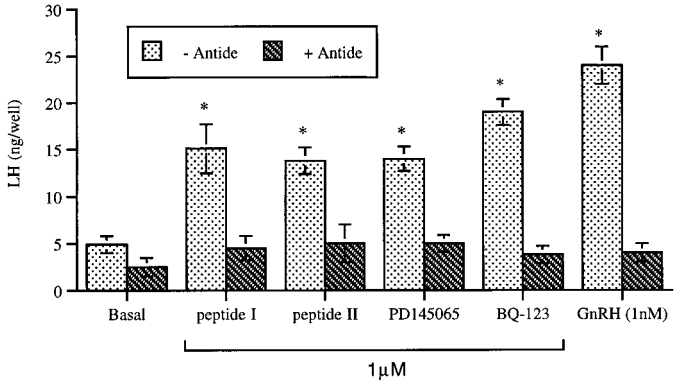

Fig. 2. Induction of LH release from dispersed rat pituitary cells by several ET antagonists (1 μM each), in the absence or presence of Antide (10 nM), a GnRH antagonist. Forty-eight hours after dispersion, the medium was changed and the cells were incubated for 4 h at 37°C with the examined peptides. The medium was collected and LH concentration was determined by RIA. Induction of LH release by GnRH (1 nM) is shown for comparison. Results are the mean ± S.E. of LH concentration in two experiments (four wells per experimental group). The LH concentration in each well was determined using triplicates. *LH release significantly higher ($P < .01$) than in the control group (basal) and the group of the respective peptide + antide.

Discussion

The sequences of peptide 1, PD145065, and BQ-123 (Table 3), have no similarity to that of GnRH. Nevertheless, we have shown that these peptides are recognized by the GnRH receptor (Table 3). Furthermore, these ET antagonists stimulate LH release, which is surprising because these peptides are expected to inhibit the ET-induced activation of LH release via the ET_A receptor (Calvo et al., 1990; Stojilkovic et al., 1992). A GnRH antagonist (Fig. 2) inhibits the LH release activity of the three ET antagonists, which suggests that this activity is mediated by the pituitary GnRH receptor; i.e., these peptides are GnRH agonists.

BQ-123 was previously shown to inhibit ET-induced LH release from dispersed pituitary cells (Samson, 1992; Kanyicska and Freeman, 1993). In one report, the ability of BQ-123 (1 μM) to induce gonadotropin secretion was evaluated. It was found to have no significant LH releasing activity (Samson, 1992), in contrast to the results presented above. However, the culture conditions of the above-mentioned study (primary culture of pituitary cells kept overnight and incubated for 1 h with the ET antagonist) are different from those described in our study (primary culture of pituitary cells kept for 48 h and incubated for 4 h with the ET antagonist). Using the culture conditions described in that report (Samson, 1992), we could not demonstrate LH release even by 1 nM GnRH (data not shown), demonstrating the superior sensitivity of the culture conditions we used for examining the LH-releasing activity of GnRH analogs.

GnRH agonists that are comparable in size with the hexapeptide ET antagonists are based on fragmentation of known, potent GnRH analogs (Sandow and Konig, 1979; Haviv et al., 1989). One of the most potent hexapeptide GnRH agonists reported is [*N*-[3-indolylpropionyl]-Ser^4, D-Trp^6, Pro^{9}NHEt]GnRH[4–9]. This hexapeptide, however, contains a bulky amino-terminal residue that makes it a mass equivalent of a heptapeptide (its mass is 1018, compared with only 886 for peptide 1 and 611 for the pentapeptide BQ-123). Its potency in inducing LH release from dispersed pituitary cells is about 1% of that of GnRH (Haviv et al., 1989). Our results using a comparable assay (Figs. 1 and 2) suggest that the potencies of the three ET antagonists are less than 0.1% of that of GnRH.

The two related hexapeptides, peptide 1 and PD145065, are derived from the carboxyl-terminal portion of ET. After NMR studies, it was suggested that BQ-123 is a mimic of that portion of ET (Peishoff et al., 1995). The common motifs in all three peptides may therefore account for their ET receptor recognition and may also be the cause of their shared ability to bind to the GnRH receptor. Therefore, additional ET antagonists may also interact with the GnRH receptor and should be screened accordingly. Nevertheless, the tripeptide

ET antagonists that were tested (BQ-610, BQ-788, FR139317), did not show significant binding to the GnRH receptor (Table 3) and did not induce LH release from dispersed pituitary cells. The design of at least one of these tripeptides (BQ-610) was based on the sequence of BQ-123 (Ishikawa et al., 1992). The absence of LH-releasing activity of these high-affinity tripeptide ET antagonists further support our conclusion that the ability of the other ET antagonists tested (peptide 1, PD145065, and BQ-123) to induce LH release is not mediated by an ET receptor but rather by direct activation of the GnRH receptor.

Our results may contribute to a better understanding of the interactions of GnRH with its receptor, especially those interactions that are crucial for receptor activation. Based on structure-activity studies, the residues considered to be most critical for GnRH agonist binding and activity, are pyroglutamic acid[1], His[2], Trp[3], and the carboxy-terminal residue of Gly-amide[10]. The basic Arg[8] of GnRH is critical for high-affinity agonist and antagonist binding (Sealfon and Millar, 1995). Among these residues, only Trp is found in all three ET antagonists (Table 3), which suggests the possibility that this common Trp residue may be involved in GnRH receptor activation. This assumption is further supported by our finding that deletion of any of the Trp residues in peptide 1 results in loss of GnRH receptor recognition.

The substitution of Asp with Asn in the sequence of peptide 1 resulted in a loss of affinity for the GnRH receptor, suggesting that the carboxylic side chain in Asp may be directly involved in receptor recognition. An Asp residue is also found in PD145065 and BQ-123, although in the latter it is a D-Asp. No negatively charged group is found in the sequence of mammalian GnRH, excluding an interaction of GnRH with its receptor similar to that suggested for peptide 1. Nevertheless, two other forms of GnRH found in lower vertebrates have a negatively charged side chain: the Lampry GnRH I form contains a Glu[6] residue (Sherwood et al., 1986), and the newly identified Tunicate GnRH I form contains an Asp[5] residue, supposedly connected via a salt bridge to a Lys[8] residue (Powell et al., 1996).

Our structure-activity relationship studies are very promising for the potential development of GnRH-related drugs, because increased specificity toward the GnRH receptor was easily obtained. Thus, peptide 2, which is the nonacetylated form of peptide 1, is almost as potent a GnRH agonist as peptide 1 (Fig. 1), but its affinity for the ET receptor is completely diminished. Peptide 2 is therefore a specific GnRH agonist and may serve as a lead compound in studies leading toward the development of a new class of potent GnRH peptides and nonpeptide derivatives. Such novel analogs may have unique advantages over the currently known GnRH analogs, such as better permeability through membranal barriers, because of their notable hydrophobicity and smaller size. It may also be possible to convert these novel GnRH agonists into antagonists, as reported previously for GnRH analog fragments of a comparable size (Haviv et al., 1989).

One of the most important implications of our findings is the possible side effects of various ET antagonists. This notion is very important because of the growing interest in ET antagonists as potential medications (Miller et al., 1993). ET analogs that interact with the GnRH receptor may have unwarranted side effects on the menstrual cycle. This possibility is highlighted by our in vivo studies with BQ-123. The affinity of BQ-123 toward the GnRH receptor (Table 3) is 2 orders of magnitude lower than its affinity for the ET_A receptor (Ihara et al., 1992). Nevertheless, BQ-123 induces a significant increase in serum LH levels in rats (Fig. 3) even at the minimal dose required for the induction of a significant ET receptor-mediated antihypertensive effect in a rat model (Mihara et al., 1994). These results may be explained by the differing mechanisms of action of BQ-123, agonistic versus antagonistic, on the GnRH and ET receptors, respectively.

TABLE 2

Inhibition of ^{125}I-[D-Lys^6]GnRH binding to its pituitary receptor by peptide 1 and related chimera

See legend to Table 1 for details. The sequence common to all peptides is in **bold**. Results are the mean ± S.E. of two experiments carried out in triplicate.

Peptide	Sequence	GnRH Receptor K_i
		μM
[D-Trp^6]GnRH	pGlu-His-Trp-Ser-Tyr-**D-Trp-Leu**-Arg-Pro-Gly-NH_2	0.0002
Ac-[D-Trp^6]GnRH[6–10]	Ac-**D-Trp-Leu**-Arg-Pro-Gly-NH_2	2.8 ± 0.4
Peptide 1	Ac-**D-Trp-Leu**-Asp-Ile-Ile-Trp-OH	1.9 ± 0.5
Chimera 1	pGlu-His-Trp-Ser-Tyr-**D-Trp-Leu**-Asp-Ile-Ile-Trp-OH	27 ± 6
Chimera 2	pGlu-His-Trp-Ser-Tyr-**D-Trp-Leu**-Asp-Ile-Ile-Trp-NH_2	4.0 ± 0.4
Chimera 3	pGlu-His-Trp-Ser-Tyr-**D-Trp-Leu**-Arg-Ile-Ile-Trp-OH	0.3 ± 0.1

pGlu, pyroglutamic acid.

TABLE 3

Binding of ET antagonists to the GnRH receptor

IC_{15}, concentration of peptide that displaces 15% of ^{125}I-[D-Lys^6]GnRH bound to rat pituitary membranes. Values are based on displacement curves obtained by incubating pituitary membranes for 90 min at 4°C with ^{125}I-[D-Lys^6]GnRH and increasing concentrations of the unlabeled peptides. Results are the mean ± S.E. of two experiments carried out in triplicates.

Peptide	Sequence	K_i	IC_{15}
		μM	μM
Peptide 1	Ac-D-Trp-Leu-Asp-Ile-Ile-Trp-OH	1.9 ± 0.5	0.4 ± 0.2
PD145065	Ac-D-Bhg-Leu-Asp-Ile-Ile-Trp-OH	8 ± 1	1.5 ± 0.6
BQ-123	Cyclo(D-Trp-D-Asp-Pro-D-Val-Leu) (Na Salt)	3.1 ± 0.6	0.7 ± 0.3
BQ-788	Dmpc-γMeLeu-D-Trp(COOMe)-D-Nle-ONa		9 ± 1
BQ-610	Hhac-Leu-D-Trp(N^{1n}CHO)-D-Trp-OH		NB
FR139317	Hhac-Leu-D-Trp(Me)-D-Ala(Pyridyl)-OH		7 ± 2

D-Bhg, D-5H-dibenzo[*a*,*d*]cycloheptene-5-acetic acid; Dmpc, *N-cis*-2,6-Dimethylpiperidinocarbonyl; Hhac, hexahydro-1H-azepinylcarbonyl. NB, no significant binding at 10 μM.

Consequently, although BQ-123 is a rather weak GnRH agonist, its GnRH agonistic activity may cause profound side effects if used for blocking the ET receptor.

The importance of our results is emphasized by the fact that BQ-123 is currently being used in clinical trials (Haynes et al., 1995; Berrazueta et al., 1997; Schmetterer et al., 1998) and may induce unwarranted side effects that are related to reproduction in both men and women. BQ-123 administered to women will induce gonadotropin secretion that may either disrupt the synchronization of the menstrual cycle or induce a gonadotropin surge that may lead to ovulation. Moreover, the effect of this drug on women who are using contraceptive pills is also a matter of concern. A long-term protocol of BQ-123 was suggested as a treatment for several indications. Thus, in a rat model designed for chronic heart failure, BQ-123 was infused by implanted minipumps at a rate of 7.5 mg/day/rat for 12 weeks (Sakai et al., 1996). Nevertheless, we have demonstrated (Fig. 3) a significant increase in serum LH concentration after the administration of a single dose of 22 μg of BQ-123/rat (100 μg/kg). The chronic administration of BQ-123 may therefore result in pituitary GnRH receptor down-regulation and desensitization, as occurs with GnRH agonists, or desensitization of the gonads as a consequence of the elevated serum concentrations of gonadotropins. In either case, desensitization leads to hyposecretion of gonadal steroids, resulting in numerous side effects such as hot flashes, decreased libido, infertility, osteoporosis, etc. (Filicori, 1994). Our findings call for a careful re-evaluation of ET antagonists, based on screening for potential GnRH agonistic activity, to prevent unwarranted side effects of ET antagonist-based drugs.

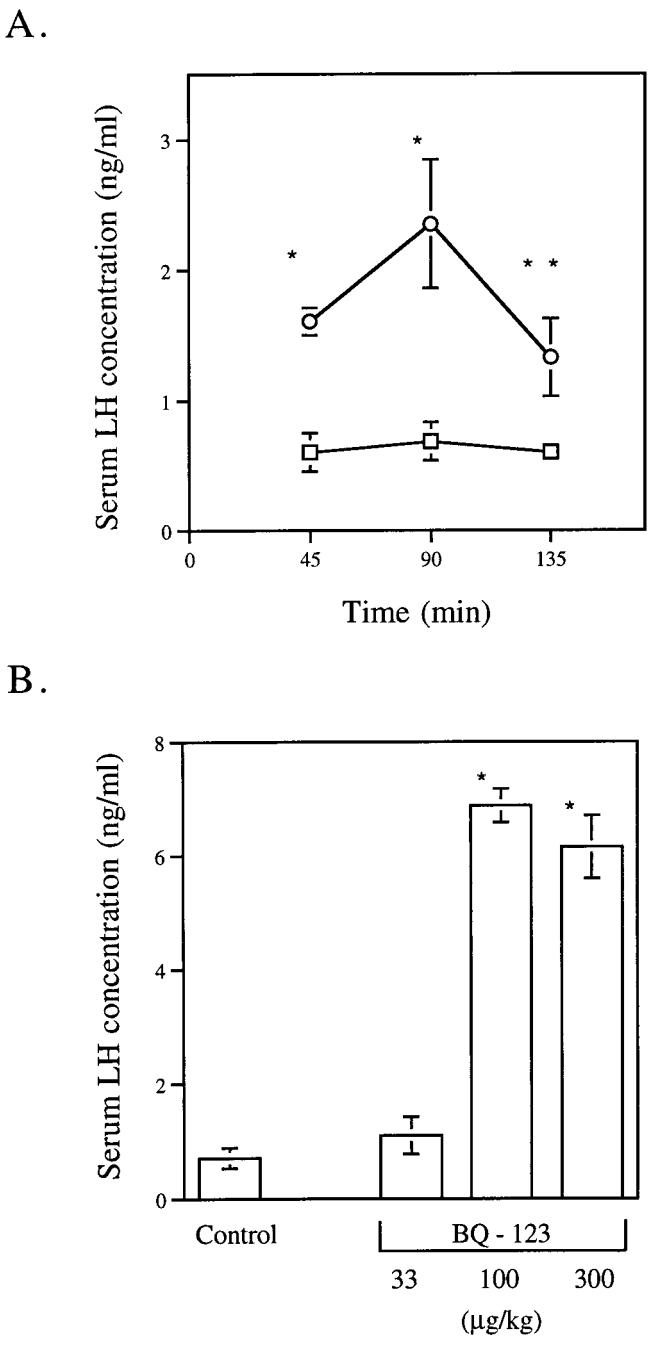

Fig. 3. Induction of LH release after i.p. administration of the ET antagonist BQ-123 to proestrous rats. A, time response. Blood samples were withdrawn at 45, 90, and 135 min after the drug administration by cardiac puncture under light ether anesthesia. □, control; ○, BQ-123. B, dose response. Three different doses of BQ-123 were administered and blood samples were withdrawn 90 min after drug administration. The serum samples were assayed for LH by using RIA. Results are the mean ± S.E. of LH concentrations in the serum of six animals per experimental group. The LH concentrations were determined using three dilutions of each serum sample. LH secretion is significantly higher (*$P <$ .001; **$P <$ .01) than in the vehicle control group.

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Send reprint requests to: Dr. Yitzhak Koch, Department of Neurobiology, Weizmann Institute of Science, Rehovot 76100, Israel. E-mail: y.koch@weizmann.ac.il