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Photoaffinity Labeling of Pituitary GnRH Receptors: Significance of the Position of Photolabel on the Ligand[†,‡]

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ABSTRACT: Photoreactive derivatives of GnRH and its analogues were prepared by incorporation of the 2-nitro-4(5)-azidophenylsulfenyl [2,4(5)-NAPS] group into amino acid residues at positions 1, 3, 6, or 8 of the decapeptide sequence. The modification of Trp[3] by the 2,4-NAPS group led to a complete loss of the luteinizing hormone (LH) releasing as well as LH-release-inhibiting activity of the peptide. The [D-Lys(2,4-NAPS)][6] analogue was a very potent agonist that, after covalent attachment by photoaffinity labeling, caused prolonged LH secretion at a submaximal rate. [Orn(2,4-NAPS)][8]-GnRH, a full agonist with a relative potency of 7% of GnRH, after photoaffinity labeling caused prolonged maximal LH release from cultured pituitary cells. In contrast, [Orn(2,5-NAPS)][8]-GnRH, although being equipotent with the 2,4-NAPS isomer in terms of LH releasing ability, was unable to cause prolonged LH release after photoaffinity labeling. Thus, [Orn(2,4-NAPS)][8]-GnRH is a very effective photolabeling ligand of the functionally significant pituitary GnRH receptor. Based on this compound, a pituitary peptidase resistant derivative, D-Phe[6],[Orn(2,4-NAPS)][8]-GnRH-(1–9)-ethylamide, was synthesized. This derivative showed high-affinity binding to pituitary membranes with a K_d comparable to those of other GnRH analogues. A radioiodinated form of this peptide was used for pituitary GnRH-receptor labeling. This derivative labeled 59- and 57-kDa proteins in rat and 58- and 56-kDa proteins in bovine pituitary membrane preparations, respectively. This peptide also labeled pituitary GnRH receptors in the solubilized state and therefore appears to be a suitable ligand for the isolation and further characterization of the receptor.

Receptors of peptide hormones can be identified and characterized and their role in mediating target cell response studied by photoaffinity labeling using suitable photoreactive ligands (Bayley & Knowles, 1977; Ramachandran et al., 1981; Eberle, 1983; Bayley, 1984). Successful application of photoaffinity labeling requires ligands with binding affinity close to the natural ligand. For direct receptor labeling it is of primary significance to select ligands whose photogenerated cross-linking groups are in optimal spatial arrangement and

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form a covalent bond with the receptor itself.

Previous attempts of photoaffinity labeling pituitary receptors of gonadotropin releasing hormone (GnRH) used derivatives carrying the photoreactive group in the side chain of D-Lys^6-substituted GnRH (Hazum, 1981; Hazum & Keinan, 1983; Nikolics et al., 1984; Iwashita & Catt, 1986). However, these analogues did not yield maximal persistent signal after photoactivation, suggesting either incomplete photoaffinity labeling or nonoptimal spatial arrangement of the covalently bound hormone. Conformational studies (Donzel et al., 1977; Cann et al., 1979; Bello et al., 1981; Kopple, 1981), conformational modeling based on energy calculations (Momany, 1976, 1978), and structure–activity studies with GnRH (Sandow et al., 1978; Morley, 1980; Rivier et al., 1981; Karten & Rivier, 1986) suggested that the spatial interaction between GnRH and its receptor requires the N- and C-terminal segments of the decapeptide, while the middle of the sequence is held away from the receptor (Momany et al., 1977). This view was further supported by the full gonadotropin-releasing activity of GnRH analogues substituted by different macromolecules, significantly larger than the decapeptide, via side chains of D-amino acids in position 6. Polyglutamic acid, M_r 100 000 (Amoss et al., 1974), polyethylene glycol, M_r 30 000 (Sandow et al., 1978), ferritin (Hopkins & Gregory, 1977) or even agarose (Conn & Hazum, 1981) could be attached to D-Lys^6- or D-Glu^6-GnRH and the resulting conjugates remained full agonists in terms of stimulating LH release. These results suggest that the side chain of a D-Lys^6 analogue is not facing the receptor and therefore does not appear to be an ideal site for receptor cross-linking or photoaffinity labeling.

In this paper the preparation and characterization of GnRH derivatives containing the photoreactive 2-nitro-4(5)-azidophenylsulfenyl [2,4(5)-NAPS] group (Muramoto & Ramachandran, 1980) in different positions of the GnRH decapeptide molecule are described. These peptides were tested in pituitary cell culture for biological activity, i.e., their ability to stimulate luteinizing hormone (LH) secretion with and without exposure to light. This functional characterization of the peptides served as a basis for developing an appropriate peptidase-resistant derivative that was successfully used for photoaffinity labeling of rat and bovine pituitary GnRH receptors in intact and solubilized membrane preparations.

Materials and Methods

Preparation of Photoreactive GnRH Derivatives. Peptides were synthesized by automated solid-phase synthesis in a Beckman 990B synthesizer. Cleavage of the peptide from the resin by liquid hydrogen fluoride and purification by gel filtration and semipreparative reverse-phase high performance liquid chromatography (HPLC) were carried out according to procedures described in detail elsewhere (Nikolics et al., 1984; Mezo et al., 1984). Purity of the NAPS derivatives of the GnRH analogues was tested by thin-layer chromatography on silica gel plates (Kieselgel 60, Merck) using the solvent system ethyl acetate–pyridine–acetic acid–water 30:20:6:11 (v/v) and by analytical reverse-phase HPLC on Vydac (Separations Group) and Synchropak (SynChrom, Inc.) C-18 columns (pore size 300 Å) using an acetonitrile–0.1% aqueous trifluoroacetic acid solvent system and monitoring the effluent at 210, 280, and 400 nm. Ultraviolet and visible spectra of the purified NAPS derivatives showed characteristic absorbance in the 400–430-nm range as described earlier (Nikolics et al., 1984).

The NAPS group can be used for tryptophan derivatization under acidic reaction conditions (Muramoto & Ramachandran, 1980) and for primary amino group derivatization under basic (Muramoto et al., 1982; Nikolics et al., 1984) reaction conditions. We took advantage of this versatility and prepared derivatives by both methods.

(*a*) *[Trp(2,4-NAPS)]^3-GnRH.* The method used for the preparation of NAPS-ACTH was applied (Muramoto & Ramachandran, 1980). GnRH (4 mg) was dissolved in 1 mL of glacial acetic acid and reacted with 4 mg of 2,4-NAPS-Cl for 1 h at room temperature in the dark. The solution was then applied onto a Sephadex LH-20 (Pharmacia) column (55 × 1.2 cm) equilibrated with methanol–dimethylformamide (DMF) 4:1 (v/v) to separate the peptide from excess 2,4-NAPS-Cl. The peptide was further purified by HPLC on a Vydac C-18 10-μm, 25 × 0.46 cm column eluting with a gradient of acetonitrile–0.02 M ammonium acetate, pH 4.0. The pooled fractions containing [Trp(2,4-NAPS)]^3-GnRH as verified by UV and visible spectra were lyophilized to give 1.2 mg of homogeneous material.

(*b*) *[Orn(2,4-NAPS)]^8-GnRH.* Orn^8-GnRH (Yabe et al., 1974) (8 mg) was reacted with 8 mg of 2,4-NAPS-Cl in the presence of 3 mg of indole in 2 mL of dimethylformamide containing 5% triethylamine (TEA) for 1 h at room temperature. The peptide was purified by gel filtration on a Sephadex LH-20 column (62 × 1.2 cm) eluting with methanol–dimethylformamide 2:1. The fraction containing the photoreactive peptide was further purified to homogeneity by reverse-phase HPLC on a Magnum-9 ODS-2, 10-μm, 25 × 0.9 cm (Whatman) column using a gradient of acetonitrile in 0.02 M ammonium acetate, pH 4.0.

The following GnRH derivatives were prepared from the corresponding synthetic peptides and purified by methods similar to that described for [Orn(2,4-NAPS)]^8-GnRH: D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-(1–9)-ethylamide; Ac-[D-Lys(2,4-NAPS)]^1,D-Cpa^2,D-Trp^3,D-Arg^6,D-Ala^{10}-GnRH; [D-Lys(2,4-NAPS)]^6-GnRH-EA.

The following peptide was prepared and purified by using analogous methods as described for the 2,4-NAPS derivatives, except in this case 2,5-NAPS-Cl (Muramoto & Ramachandran, 1980) was used in an equal amount with Orn^8-GnRH for the reaction: [Orn(2,5-NAPS)]^8-GnRH.

Preparation of Radioiodinated Photoreactive GnRH Derivatives. For experiments with pituitary membrane preparations to label the GnRH receptor, radioiodinated derivatives were prepared. Since the direct radioiodination of the NAPS derivatives described above by various methods was found to result in complex mixtures difficult to purify, probably due to partial iodination of the NAPS group, a different approach was used. The peptides were first radioiodinated and then derivatized with 2,4-NAPS-Cl.

(*a*) *^{125}I-Tyr^5,D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-(1–9)-ethylamide.* To 10 μg of D-Phe^6,Orn^8-GnRH-(1–9)-ethylamide (EA) dissolved in 20 μL of phosphate buffer, 0.5 M, pH 7.4, were added approximately 2 mCi of Na^{125}I and 0.5 μg of chloramine T in 5 μL of buffer, and the mixture was reacted for 40 s. The reaction was stopped by the addition of 10 μg of sodium metabisulfite. The radioiodinated peptide was purified by reverse-phase HPLC on a Vydac C-18, 5-μm, 0.46 × 250 mm column by a gradient of 0–40% acetonitrile–0.1% trifluoroacetic acid (TFA) at a rate of 1%/min and a flow rate of 1 mL/min. Aliquots of the 1-mL fractions were counted in a γ-counter, and the peptide peak was collected. Specific activity of the purified peptide was approximately 1000 Ci/mmol as determined by self-displacement in the binding assay. After the addition of dimethylformamide to this solution, water was removed under vacuum. 2,4-

NAPS-Cl (20 μg) and indole (4 μg) in DMF–2% triethylamine were added, and the mixture was reacted for 4 h. The resulting photoreactive derivative was purified by reverse-phase HPLC as described above except the gradient applied was 10–60% acetonitrile in 0.1% aqueous TFA. The peak fractions were pooled and diluted to a radioactivity concentration of approximately 100 000 cpm/mL with methanol–1% TEA and stored at 4 °C until use when methanol was removed in vacuo. Under these conditions the peptides could be used for photoaffinity labeling for 2 weeks. Specific activity of the peptide was not altered by the derivatization.

(b) ^{125}*I-Tyr*^{5},[D-*Lys(2,4-NAPS)*]^6-*GnRH-(1–9)-ethylamide.* This peptide was prepared by methods similar to that described above for D-Lys^6-GnRH-(1–9)-ethylamide.

Gonadotropin-Releasing Activity of Photoreactive GnRH Analogues. Primary cultures of rat anterior pituitary cells from pituitaries of Sprague-Dawley female rats (180–220 g) (Simonsen Labs, Gilroy, CA) were prepared by the method described previously (Nikolics et al., 1981). Anterior pituitaries were dispersed by trypsin treatment and after several washes with Medium 199 (Gibco, with Earle's salts, $NaHCO_3$, and glutamine) were placed into 24 multiwell culture plates (Costar). Each well contained approximately 5×10^5 cells in 1 mL of Medium 199 supplemented with 10% fetal bovine serum (Hyclone). Cells were cultured at 37 °C in 5% CO_2 atmosphere and maximal humidity. The medium was changed on the third or fourth day, and experiments were carried out on the fourth or fifth day in quadruplicate culture wells for each experimental point.

For the determination of the intrinsic LH-releasing ability of the photoreactive analogues, these were incubated at different concentrations with the cultured cells for 4 h at 37 °C in Medium 199 without serum. In experiments testing for inhibition of LH release, 10^{-7}–10^{-9} M concentrations of the competitive inhibitory analogue Ac-D-Trp^{1,3},D-Cpa^2,D-Lys^6,D-Ala^{10}-GnRH (Mezo et al., 1984; Kovacs et al., 1984) and 10^{-9} M of the agonist D-Phe^6-GnRH-EA (Nikolics & Spona, 1985) were added simultaneously to culture wells. Similarly, [Trp(2,4-NAPS)]^3-GnRH, when tested for its possible inhibitory activity, was incubated together with D-Phe^6-GnRH-EA with pituitary cells for 4 h, and LH was measured in aliquots of the media.

LH was determined in duplicate in aliquots of culture media using a double-antibody radioimmunoassay with materials (rat LH RIA kit) provided by Dr. A. F. Parlow through the National Hormone and Pituitary Program, NIDDK. Results are expressed in terms of the LH-RP-2 reference preparation.

Photoaffinity Labeling Experiments with Pituitary Cell Cultures. The effect of covalent linking of GnRH analogues to intact, functional rat pituitary cells was evaluated by using photoaffinity labeling under conditions that did not interfere with cell viability and gonadotropin release. The photoreactive analogues were first incubated with pituitary cells at 37 °C in Medium 199 without serum for 20 min in the dark. Then the multiwell plates were irradiated with a Blak-Ray long-wave UV lamp (emission wavelength 365 nm) for 2×5 min at 25–28 °C from a distance of 10 cm. Between the two UV exposures medium was removed and medium containing fresh photoreactive peptide was added. Such conditions have been found to be optimal for photoaffinity labeling of cultured pituitary cells with [D-Lys(NAPS)]^6-GnRH-EA (Nikolics et al., 1984) and adrenal cells with NAPS-ACTH (Ramachandran et al., 1981). Anterior pituitary cells remained intact and fully responsive to GnRH after 2×5 min of UV exposure as verified by controls. "Dark controls" were tested identically; however, these multiwell plates were not exposed to UV light and therefore resulted in no covalent attachment of the photoreactive ligands. Prephotolyzed peptides were also used as controls that did not bind covalently to the cells as shown earlier (Nikolics et al., 1984). After irradiation, cells were washed with 4×1 mL of medium during a period of 20 min and incubated in fresh medium for 4 h in the dark unless otherwise indicated. LH released into the medium was determined as described by RIA. This sequence of incubations and washes allows the measurement of gonadotropin release stimulated only by covalently bound GnRH analogues.

Experiments with Pituitary Membrane Preparations. Plasma membranes of rat and bovine anterior pituitaries were prepared by slight modifications of described methods (Clayton et al., 1979a,b; Perrin et al., 1983). Anterior pituitaries from 60 adult male Sprague-Dawley rats (250–275 g) (Simonsen Labs, Gilroy, CA) were homogenized in 6 mL of 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM phenylmethanesulfonyl fluoride, and 0.1% bacitracin, pH 8.0, in a Teflon–glass homogenizer. The homogenate was centrifuged at 800*g* for 10 min and the resulting supernatant at 30000*g* for 60 min. The resulting pellet ("crude membranes") was resuspended in 10 mM Tris-HCl, pH 7.5, and 5% glycerol and used for binding displacement and photoaffinity labeling experiments.

Bovine pituitaries (freshly obtained through JR Scientific, Woodland, CA) were dissected to remove the posterior lobe and connective tissue. The minced anterior pituitaries were homogenized in 20 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, and 0.1% bacitracin, pH 8.0, by an Ultra-Turrax homogenizer (Janke-Kunkel) and centrifuged at 600*g* for 10 min. The supernatant was centrifuged at 25000*g* for 90 min. The upper pink layer of the pellet that showed high-affinity binding was homogenized in a Teflon–glass homogenizer in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.4, and layered onto a discontinuous sucrose density gradient (5 mL each of 1.12, 1.14, 1.16, 1.18 and 1.20 density sucrose) and centrifuged for 14 h. The middle layer, 1.16 density, which showed the highest binding activity, was diluted and homogenized in a Teflon–glass potter with 10 mM HEPES buffer, pH 7.4, and centrifuged at 30000*g* for 90 min. Other layers showed significantly less specific binding activity and were not used for these experiments. The resulting pellet ("purified membranes") was used for binding and photoaffinity labeling experiments. An aliquot of this preparation was solubilized in 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)/10 mM HEPES/10% glycerol/0.5 mM dithiothreitol (DTT) (Perrin et al., 1983) by homogenization in a Teflon–glass homogenizer. The mixture was centrifuged at 100000*g* for 75 min and the supernatant used for photoaffinity labeling experiments.

Binding experiments were carried out according to the methods of Clayton et al. (1979b) and Conne et al. (1979).

Photoaffinity labeling of pituitary membrane preparations was carried out by methods similar to those described by Hazum (1981) and Iwashita and Catt (1986). Pituitary membranes (400 μg of rat and 800 μg of bovine) were incubated in glass tubes with the photoreactive, radioactive derivatives (approximately 400 000 cpm) for 80 min at 4 °C in the dark. Then the tubes were exposed to long-wave UV light (Blak-Ray, long-wave UV lamp, 365-nm primary emission) while the mixtures stirred at 4 °C for 8 min. The membranes were centrifuged through a layer of 0.1% BSA/10 mM Tris-HCl, pH 7.4, and then resuspended in fresh buffer and

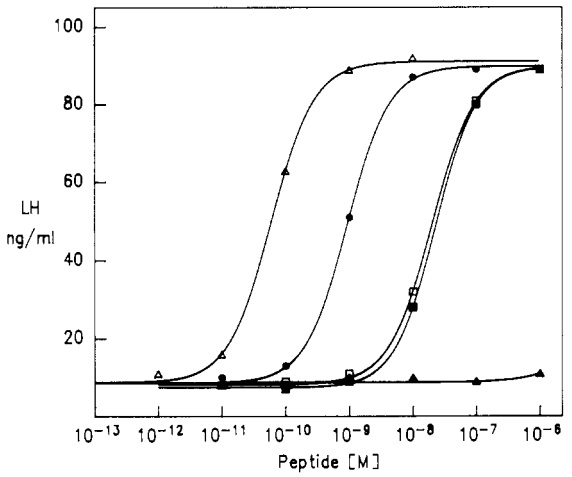

FIGURE 1: Effect of GnRH and the photoreactive GnRH derivatives on LH release from rat anterior pituitary cells in culture. Increasing concentrations of GnRH (●), [Trp(2,4-NAPS)]^3-GnRH (▲), [D-Lys(2,4-NAPS)]^6-GnRH-EA (△), [Orn(2,4-NAPS)]^8-GnRH (□), or [Orn(2,5-NAPS)]^8-GnRH (■) were added to 5×10^5 cells in monolayer cell culture and incubated for 4 h. LH released into the medium was measured by RIA and is expressed in terms of the NIADDK reference (LH-RP-2) in ng $(5 \times 10^5$ cells$)^{-1}$.

centrifuged repeatedly. The pellet was solubilized in 2% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, pH 7.0, 10% glycerol, and 2% β-mercaptoethanol and boiled for 3 min. Aliquots of this solution were analyzed by polyacrylamide gel electrophoresis (PAGE) on 10% SDS gels (Laemmli, 1970). The gels were dried onto filter paper and placed onto Kodak XAR-2 film for various periods and developed. Photoaffinity labeling of solubilized bovine pituitary membranes was carried out similarly except aliquots of the UV-exposed mixture were directly analyzed by SDS–PAGE.

Results

Photoreactive derivatives of GnRH that carry the 2-nitro-4(5)-azidophenylsulfenyl group in different positions of the peptide have been prepared by use of 2,4(5)-NAPS-Cl, which enables selective modifications of tryptophan under acidic reaction conditions (Muramoto & Ramachandran, 1980) and free amino groups under slightly basic reaction conditions (Muramoto et al., 1982; Nikolics et al., 1984). GnRH derivatives that contained the NAPS group in positions 1, 3, 6, or 8 of the decapeptide sequence have been prepared and purified to homogeneity. These peptides were evaluated for their gonadotropin-releasing and release-inhibiting activities in rat anterior pituitary cell cultures. For labeling of GnRH receptors in rat and bovine anterior pituitary membrane preparations, radioiodinated forms of two of the peptides, D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-EA and [D-Lys(2,4-NAPS)]^6-GnRH-EA were prepared and purified.

Under the irradiation conditions used in the experiments described, all derivatives could be fully photolyzed within 30–40 min. Within 5 min of exposure to long-wave UV light, the derivatives showed approximately 50% photolysis (Nikolics et al., 1984). This period of irradiation, repeated with freshly added photoreactive peptides, was found to cause maximally efficient photoaffinity labeling in terms of prolonged LH release in pituitary cell cultures and efficient labeling of pituitary membrane preparations. The most optimal preincubation time with the photoreactive analogues before UV exposure was found to be 15–20 min at 37 °C for cell cultures, 70–80 min at 4 °C for membrane preparations, and 15–20 min at 25 °C for solubilized receptor preparations.

The derivatives exhibited diverse LH-releasing activities in rat pituitary cell cultures (Figure 1). [Trp(2,4-NAPS)]^3-GnRH was completely inactive in concentrations up to 10^{-7} M. [D-Lys(2,4-NAPS)]^6-GnRH-EA was a highly active agonist of GnRH in terms of stimulating LH release with a relative potency of approximately 18-fold compared to GnRH itself, yet it was 4-fold less active than the parent molecule, D-Lys^6-GnRH-EA (not shown). The two NAPS derivatives of Orn^8-GnRH were equipotent, and both were full agonists with a relative potency of approximately 0.06–0.08. The parent peptide, Orn^8-GnRH had a relative activity of 0.6 (not shown); therefore, the introduction of the NAPS group caused an approximately 8–10-fold decrease in gonadotropin-releasing activity due to the loss of charge in the side chain of position 8 and possible spatial reasons.

Table I: Effect of [Trp(2,4-NAPS)]^3-GnRH on LH Release from Cultured Pituitary Cells Induced by D-Phe^6-GnRH-(1–9)-ethylamide

[[Trp(2,4-NAPS)]^3-GnRH] (M)	[D-Phe^6-GnRH-EA] (M)	LH release [ng $(5 \times 10^5$ cells$)^{-1}$ 4 h^{-1}]
		12.2 ± 1.7
	10^{-9}	102.5 ± 10.0
10^{-9}		10.8 ± 1.5
10^{-7}		10.3 ± 2.1
10^{-9}	10^{-9}	109.1 ± 12.7
10^{-7}	10^{-9}	121.4 ± 14.2
[inhibitory analogue][a] (M)	[D-Phe^6-GnRH-EA] (M)	LH release [ng $(5 \times 10^5$ cells$)^{-1}$ 4 h^{-1}]
10^{-9}	10^{-9}	22.7 ± 2.8
10^{-8}	10^{-9}	9.6 ± 2.0
10^{-7}	10^{-9}	11.8 ± 1.8

[a] The inhibitory analogue used as control was Ac-D-Trp^{1,3},D-Cpa^2,D-Lys^6,D-Ala^{10}-GnRH.

Since [Trp(2,4-NAPS)]^3-GnRH was completely inactive, it was also tested as a potential inhibitory analogue of GnRH as shown in Table I. However, the peptide was unable to inhibit the stimulatory effect of the agonist, D-Phe^6-GnRH-EA in a concentration ratio of 100:1, whereas a potent GnRH antagonist used as control caused full inhibition of LH release in even lower concentrations, suggesting that the bulky NAPS group attached to the indole side chain interfered with the ability of this derivative to bind to GnRH receptors.

After photolysis, [Trp(2,4-NAPS)]^3-GnRH in low concentrations remained inactive; however, at higher concentrations (10^{-7} and 10^{-6} M) it showed a low level of LH-releasing activity (Figure 2). The [Trp(2,4-NAPS)]^3-GnRH analogue was also tested for its ability to inhibit the gonadotropin-releasing effect of D-Phe^6-GnRH-EA following photolysis (Table II). However, no inhibition could be observed, whereas the photoreactive GnRH antagonist Ac-[D-Lys(2,4-NAPS)]^1,D-Cpa^2,D-Trp^3,D-Arg^6,D-Ala^{10}-GnRH used as control at the same concentration ratio caused very significant inhibition.

[D-Lys(2,4-NAPS)]^6-GnRH-EA was able to form a covalent link to the surface of pituitary gonadotrophs following exposure to UV light as indicated by prolonged stimulation of LH release in a dose-dependent manner (Figure 2). The maximal effect elicited during the 4-h incubation, however, was lower than that induced by 10^{-8} M GnRH, which was added to the incubation medium after the cells were exposed to ultraviolet light.

The [Orn(2,4-NAPS)]^8-GnRH analogue after photoaffinity labeling caused persistent activation of LH release at a maximal rate comparable to that caused by 10^{-8} M GnRH, suggesting that the derivative formed a covalent link with the receptor in a relatively optimal manner. In surprising contrast, [Orn(2,5-NAPS)]^8-GnRH after photolysis did not release LH

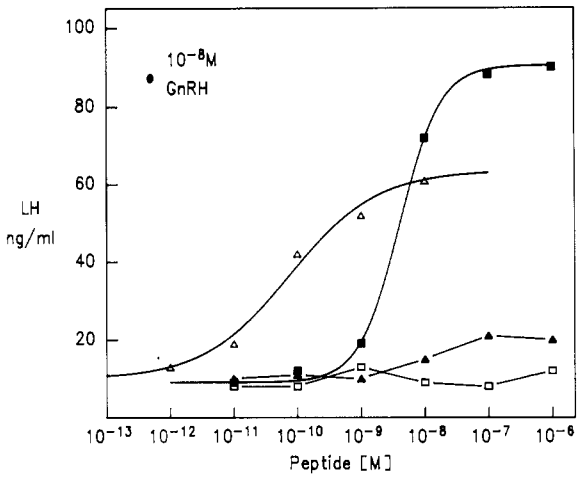

FIGURE 2: Persistent LH release induced by [Trp(2,4-NAPS)]^3-GnRH (▲), [D-Lys(2,4-NAPS)]^6-GnRH-EA (△),[Orn(2,4-NAPS)]^8-GnRH (■), or [Orn(2,5-NAPS)]^8-GnRH (□) during 4 h of incubation following photoaffinity labeling. The peptides were added to cultured rat pituitary cells (5×10^5) and incubated for 20 min and then exposed to UV light for 2 × 5 min as described under Materials and Methods. The values shown represent LH released into the medium by covalently bound GnRH derivatives. LH was determined by RIA and is expressed as LH-RP-2, ng (5×10^5 cells)$^{-1}$. The value on the left (●) represents secretion stimulated by 10^{-8} M GnRH during the 4-h incubation period following UV exposure of the culture.

Table II: Effect of [Trp(2,4-NAPS)]^3-GnRH after Photolysis on LH Release from Cultured Pituitary Cells Induced by D-Phe^6-GnRH-(1–9)-ethylamide

[[Trp(2,4-NAPS)]^3-GnRH] (M), preincubation and photolysis	[D-Phe^6-GnRH-EA] (M), incubation	LH release [ng (5×10^5 cells)$^{-1}$ 4 h^{-1}]
	10^{-9}	108.2 ± 10.5
10^{-9}		11.8 ± 2.2
10^{-7}		21.3 ± 2.6
10^{-9}	10^{-9}	128.1 ± 13.4
10^{-7}	10^{-9}	142.6 ± 28.6
[photoreactive inhibitor] (M)	[D-Phe^6-GnRH-EA] (M)	LH release [ng (5×10^5 cells)$^{-1}$ 4 h^{-1}]
10^{-7}		18.7 ± 4.4
10^{-9}	10^{-9}	36.8 ± 3.2
10^{-7}	10^{-9}	39.1 ± 5.9

[a] The inhibitory analogue used as control was Ac-[D-Lys(2,4-NAPS)]^1,D-Cpa^2,D-Trp^3,D-Arg^6,D-Ala^{10}-GnRH. Experimental details are given under Materials and Methods.

even at high concentrations (Figure 2). Due to degradation by pituitary peptidases (Clayton et al., 1979a), no direct receptor binding could be measured with derivatives containing Gly in position 6. To determine an approximate value of fractional receptor occupancy by the different covalently bound peptides, after photoaffinity labeling with all the analogues, cells were also incubated with a maximally effective concentration (10^{-8} M) of GnRH. The sum of the two values of LH released during the first (photolabeling) and second (GnRH) challenge was found to be constant; i.e., no LH could be released after photoaffinity labeling with [Orn(2,4-NAPS)]^8-GnRH, approximately 50% after [D-Lys(2,4-NAPS)]^6-GnRH-EA, and maximal amounts after [Orn(2,5-NAPS)]^8-GnRH (not shown).

Peptides that had been prephotolyzed before incubation and photolysis with the cells did not cause prolonged LH release. Covalent attachment of the effective photoreactive peptides, the [D-Lys(2,4-NAPS)]^6-GnRH-EA and the [Orn(2,4-NAPS)]^8-GnRH analogues, was further verified by the use

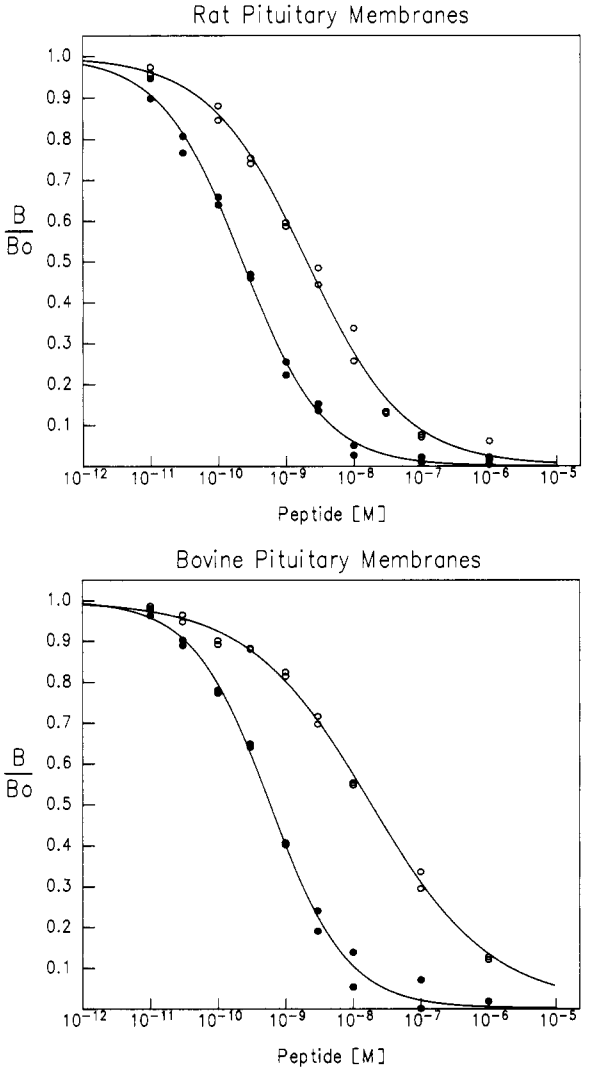

FIGURE 3: Inhibition of binding of ^{125}I-D-Phe^6,Orn^8-GnRH-EA to rat and bovine pituitary membrane preparations by increasing concentrations of D-Phe^6,Orn^8-GnRH-EA (●) or D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-EA (○). The indicated concentrations of the peptides were incubated with rat or bovine pituitary membrane preparations as described under Materials and Methods. Specifically bound radioactivity is expressed as a function of increasing concentrations of the displacing peptides.

of a potent competitive inhibitor of GnRH. This analogue (Ac-D-Trp^{1,3},D-Cpa^2,D-Lys^6,D-Ala^{10}-GnRH) was added to cell cultures photolyzed previously with the two photoreactive analogues, yet no inhibition could be observed (data not shown).

On the basis of these results an analogue was synthesized that contained a D-amino acid substitution in position 6, in order to prevent degradation by pituitary membrane-bound peptidases, and ornithine in position 8 for the introduction of the 2,4-NAPS group. This derivative, D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-EA, showed high-affinity binding to rat pituitary membrane preparations ($K_d = 2 \times 10^{-9}$ M) as shown in Figure 3, although the introduction of the NAPS group into the ornithine^8 side chain led to a significant decrease in binding affinity: approximately 10-fold in the case of rat membranes and 15-fold in the case of bovine membranes. This decrease was similar to the decrease in activity seen with rat pituitary cell cultures (Figure 1).

A radioiodinated form of this peptide was used for photoaffinity labeling of rat and bovine pituitary membranes followed by solubilization and SDS–polyacrylamide electrophoretic analysis of membrane proteins. As shown in Figure

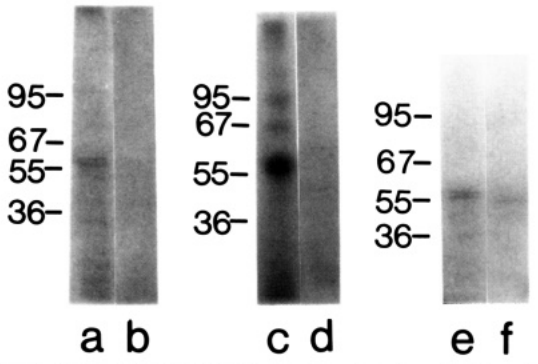

FIGURE 4: Photoaffinity labeling of rat and bovine pituitary GnRH receptors. (a, b) Labeling of crude rat pituitary membranes (800 μg of protein) after preincubation and UV exposure with ^{125}I-D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-EA (400 000 cpm) in the absence (a) and presence (b) of 10^{-6} M cold D-Phe^6-GnRH-EA. (c, d) Labeling of purified and solubilized bovine pituitary membranes with ^{125}I-D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-EA in the absence (c) and presence (d) of cold D-Phe^6-GnRH-EA. (e, f) Comparison of photoaffinity labeling of purified bovine pituitary membranes with ^{125}I-D-Phe^6,-[Orn(2,4-NAPS)]^8-GnRH-EA (e) or ^{125}I-[D-Lys(2,4-NAPS)]^6-GnRH-EA (f). Molecular weight size markers are indicated along each pair of lanes, which were run separately. Further experimental conditions are given under Materials and Methods.

4, two close protein bands, 59 000 and 57 000, were specifically labeled with this peptide (lane a). Similarly, in bovine pituitary membrane preparations a 58- and a 56-kDa protein were labeled specifically. Bovine membranes were also solubilized in CHAPS and labeled with the photoreactive peptide, resulting in intense labeling of the two close bands (lane c). In this preparation, bovine serum albumin (67 kDa) and another protein (95 kDa) were also labeled to a low degree. Photoaffinity labeling with radioiodinated isomers of both D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-EA and [D-Lys(2,4-NAPS)]^6-GnRH-EA were also compared (lanes e and f). The former peptide carrying the photoreactive group in position 8 resulted in labeling both protein bands, while the peptide with the photoreactive group in position 6 only labeled the lower band. In addition, significantly more intense labeling was observed with the Orn^8 photolabel.

Discussion

Gonadotropin-releasing hormone stimulates the release of luteinizing hormone and follicle-stimulating hormone (FSH) from pituitary gonadotrophs. The sequence of reactions leading to the secretion of LH and FSH begins with the specific recognition of GnRH by receptors on the surface of gonadotrophs. According to pituitary plasma membrane receptor binding studies, these cells possess one class of high-affinity (K_D approximately 10^{-9} M) binding sites (Clayton & Catt, 1981).

Photoaffinity labeling has been employed for labeling pituitary GnRH receptors using derivatives of the decapeptide. GnRH analogues substituted with D-amino acids in position 6 have long been known to be highly active and resistant to degradation by peptidases (Clayton et al., 1979b). Therefore, D-Lys^6-GnRH offered a possibility for the introduction of arylazide type photoreactive groups (Hazum, 1981; Nikolics et al., 1984; Iwashita & Catt, 1986). However, as suggested by conformational models (Momany, 1976; Donzel et al., 1977; Cann et al., 1978; Bello et al., 1981) and [D-amino acid^6-GnRH] macromolecular conjugates (Amoss et al., 1974; Hopkins & Gregory, 1977; Sandow et al., 1978; Conn & Hazum, 1981), the side chain of D-Lys^6-GnRH or its nonapeptide ethylamide analogues is not an optimal choice for the introduction of a photoreactive groups to achieve direct receptor labeling because the middle region of GnRH appears to be held away from the receptor. Rather, derivatives of the decapeptide carrying the photoreactive group in the N- and C-terminal regions would be more promising for this purpose.

The analogues described in the present study contained the photoreactive group in different amino acid side chains of the decapeptide, positions 3, 6, or 8. Also, an inhibitory peptide that was substituted with the NAPS group in position 1 was investigated.

The GnRH molecule is very sensitive to changes in Trp^3 (Sandow et al., 1978), which appears to play a key role in the receptor binding and effector function of the peptide. However, for the same reason, an analogue attached to the receptor via this amino acid side chain would be an ideal ligand for labeling the receptor. Yet, [Trp(2,4-NAPS)]^3-GnRH remained completely inactive even at high concentrations (Figure 1). Since this peptide did not act as a competitive inhibitor of GnRH either (Table I), we conclude that the substitution of the indole side chain by the NAPS group led to a virtually complete lack of recognition by the receptor, probably through a local (Trp^3) or overall influence on the conformation of this peptide.

Substitutions of Arg^8 also lead to analogues with decreased LH-releasing activity (Sandow et al., 1978). Yet, certain changes including acetyl-, benzyl-, or unsubstituted ornithine result in analogues that show low potency (2–12%) but full agonist activity (Yabe et al., 1974). Similarly, the NAPS derivatives of Orn^8-GnRH also had maximal LH-releasing activity (Figure 1) with a reduced relative potency of 6–8% probably due to loss of the charged side chain and simultaneous introduction of a bulky hydrophobic group. Thus, relatively higher concentrations of [Orn(2,4-NAPS)]^8-GnRH were required to release LH at maximal rates after photoaffinity labeling as shown in Figure 2. This result suggests that the peptide formed a covalent bond with the receptor in a conformationally favorable manner that led to maximal stimulation of gonadotropin secretion. The inability of the [Orn-(2,5-NAPS)]^8-GnRH analogue, despite minimal structural difference compared to the 2,4-NAPS analogue, to release LH from pituitary cells (Figure 2) suggests that a very delicate conformational fit is required for receptor cross-linking and the position of the azido group is a critical factor for photoaffinity labeling.

The results obtained with the GnRH derivatives containing the photoreactive group in positions 3 and 8 suggest that the N-terminal region is more critical for the effector function. It is pertinent to note that avian and teleost gonadotropin-releasing hormones (Miyamoto et al., 1982, 1984; King & Millar, 1982; Sherwood et al., 1983, 1986) show species differences in the C-terminal region, predominantly in position 8. Despite these differences, the peptides show low but full agonist LH-releasing ability across species (Millar & King, 1983; Miyamoto et al., 1984). In fact, the "chicken II" form containing an aromatic side chain in position 8 (His^5,Trp^7,Tyr^8-GnRH) was more potent in rat pituitary cell cultures than the other forms (Miyamoto et al., 1984). Thus, it seems that the N-terminal segment of the decapeptide is conserved for the stimulation of LH release, whereas the C-terminal sequence contributes to a preferred binding specific specific for the species. The low potency but full agonist activity of the Orn(NAPS)^8 analogues also containing an aromatic substitution in position 8 is in agreement with these data.

[D-Lys(2,4-NAPS)]^6-GnRH-EA caused significant LH secretion after photoaffinity labeling, showing that the ana-

logue formed a covalent link to the cell surface of pituitary gonadotrophs. The "immunity" of the D-amino acid[6] side chain to any substitution, however, strongly suggests that the photolabel in this position may not link directly to the receptor but some other structure in the vicinity of the receptor. This link probably maintains the molecule in a spatial arrangement that enables it to trigger the LH-releasing mechanism. In addition, even high concentrations of [D-Lys(2,4-NAPS)]^6-GnRH-EA caused significantly lower rate of LH release than [Orn(2,4-NAPS)]^8-GnRH.

On the basis of these results combined with information on GnRH structure–activity studies and conformational results, [Orn(2,4-NAPS)]^8-GnRH or related peptides with higher potency appear to be optimal photoreactive ligands for the labeling of pituitary GnRH receptors. Since [Orn(2,4-NAPS)]^8-GnRH was degraded by pituitary peptidases during incubation with membrane preparations, a degradation-resistant derivative, D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-EA, was synthesized and used for membrane binding and labeling experiments. This analogue displayed high-affinity binding with a K_d of about 10^{-8} M with both rat and bovine pituitary membrane preparations (Figure 3).

Two proteins of close but different size were found to be labeled by the radioactive photoreactive peptide, which was seen consistently in different experiments. Similar size bands were reported earlier using photoreactive GnRH analogues carrying the photoreactive group in the position 6 side chain (Hazum, 1981; Iwashita & Catt, 1986; Hazum et al., 1986). However, protein bands labeled with D-Lys^6-GnRH derivatives were diffuse and could not be clearly identified. Using radiolabeled D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-(1–9)-EA as ligand for photoaffinity labeling resulted in clear protein bands as compared to photolabeling with D-Lys^6-GnRH-(1–9)-EA derivatives [compare Figure 4 with the results of Iwashita and Catt (1986) and Hazum et al. (1986)]. In addition, the D-Lys^6 derivative appeared to label only the smaller of the two bands that were labeled by the Orn^8 derivative. In case D-Lys^6-GnRH-(1–9)-EA forms a covalent bond with a nonreceptor protein, the real receptor would be the larger (58 or 59 kDa) component. The size of the bovine GnRH receptor on the basis of our data is 58 and 56 kDa, which is different from that (42 kDa) obtained by using the D-Lys^6 derivative (Iwashita & Catt, 1986). In one of four experiments we also observed an approximately 40-kDa protein labeled with low intensity that could represent a degradation product of one of the larger proteins. The rat receptor appears to contain 59- and 57-kDa proteins. This is in agreement with the size of the purified rat pituitary GnRH receptor described recently, i.e., 59 and 57 kDa (Hazum et al., 1986), and substantiates the possibility that the intact receptor is a dimer (Conn & Venter, 1985). This receptor molecule could be composed of independent subunits forming a heterodimer or of identical subunits forming a homodimer, in which case the lower molecular size form would be a degradation or possibly a differently glycosylated product of the higher.

In conclusion, a photoreactive derivative of GnRH has been developed that is suitable for labeling the functionally relevant pituitary receptor in cell culture, in membrane preparation, and in the solubilized form, and therefore it can be used for the isolation and characterization of the pituitary GnRH receptor.

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Registry No. GnRH (porcine), 33515-09-2; [Trp(2,4-NAPS)]^3-GnRH, 112440-52-5; 2,4-NAPS-Cl, 66365-13-7; [Orn-(2,4-NAPS)]^8-GnRH, 112459-54-8; Orn^8-GnRH, 39064-78-3; D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-(1–9)-ethylamide, 112459-55-9; Ac-[D-Lys(2,4-NAPS)]^1,D-Cpa^2,D-Trp^3,D-Arg^6,D-Ala^{10}-GnRH, 97242-34-7; [D-Lys(2,4-NAPS)]^6-GnRH, 112440-53-6; 2,5-NAPS-Cl, 73784-43-7; D-Phe^6,Orn^8-GnRH, 112440-56-9; [Orn(2,5-NAPS)]^8-GnRH, 112459-56-0; Ac-D-Lys^1,D-Cpa^2,D-Trp^3,D-Arg^6,D-Ala^{10}-GnRH, 112459-57-1; D-Lys^6-GnRH, 71779-17-4; ^{125}I-Tyr^5,D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-(1–9)-ethylamide, 112440-54-7; I-Tyr^5,D-Phe^6,[Orn(2,4-NAPS)]^8-GnRH-(1–9)-ethylamide, 112459-58-2; ^{125}I-Tyr^5,[D-Lys(2,4-NAPS)]^6-GnRH-(1–9)-ethylamide, 112440-55-8; I-Tyr^5,[D-Lys(2,4-NAPS)]^6-GnRH-(1–9)-ethylamide, 112440-57-0; GnRH, 9034-40-6.

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Structure and Dynamics of α-Tocopherol in Model Membranes and in Solution: A Broad-Line and High-Resolution NMR Study†

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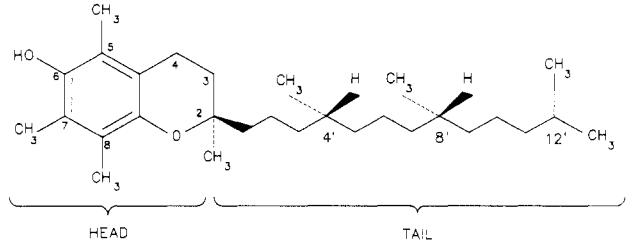
ABSTRACT: Nuclear magnetic resonance has been applied to study the conformational dynamics of α-tocopherol (vitamin E) in solution and in model membranes. In nonviscous solution, ^{1}H nuclear magnetic resonance (NMR) showed that α-tocopherol is in rapid equilibrium between two or more puckered conformers of its heterocyclic ring. The most likely conformers to be so involved are the two half-chair forms. Deuterium NMR spectra of specifically deuteriated α-tocopherol in multilamellar dispersions of egg phosphatidylcholine, measured in the liquid-crystalline state, were characteristic of axially symmetric motional averaging. The orientation of the rotational axis within the molecular framework was determined. Studies on oriented multilamellar membranes revealed that this axis is perpendicular to the surface of the membrane. The profile of quadrupolar splittings along the hydrophobic tail does not have a plateau, in contrast to that of the fatty acyl chains of the membrane lipids. Longitudinal relaxtion times (T_1) were short. The presence of a minimum in their temperature dependence shows that molecular motion with an effective correlation time $\tau_{eff} \approx 3 \times 10^{-9}$ s is responsible for relaxation. However, the temperatures and absolute values of the minima depend on the position of the deuterium in the molecule, demonstrating that τ_{eff} represents a complex blend of motions.

It is generally accepted that the major biological role of vitamin E is as a chain-breaking antioxidant, preventing peroxidation of the highly unsaturated fatty acids in membrane lipids (Burton & Ingold, 1986). Vitamin E is composed of a class of tocopherols, of which α-tocopherol (Scheme I) is the most abundant and the most active (Burton & Ingold, 1986).

Scheme I



Although a lot is known about the biological function of tocopherol, studies of its behavior in the membrane at the molecular level are rather limited. Most of the physicochemical work on model membranes with incorporated vitamin E

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