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GONADOTROPIN RELEASING-HORMONE RECEPTORS: PHOTOAFFINITY LABELING WITH AN ANTAGONIST

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A photoaffinity antagonist of gonadotropin releasing hormone (GnRH), D pGlu-D-Phe-D-Trp-Ser-D-Lys^6(N^ϵ-azidobenzoyl)-Leu-Arg-Pro-Gly-NH_2 (photoaffinity antagonist) was prepared by reacting [D-pGlu^1, D-Phe^2,D-Trp^3,D-Lys^6]GnRH with the N-hydroxysuccinimide ester of 4-azidobenzoic acid. The analog appeared homogeneous when analyzed by thin-layer chromatography and its photoreactivity was demonstrated by spectral changes when exposed to light. The photoaffinity antagonist retained high affinity binding to the GnRH receptor of pituitary membrane preparations and exhibited antagonistic activity when assayed in vitro in whole pituitaries. Pituitary membrane preparations were incubated with the radioactive photoaffinity GnRH antagonist and irradiated with light. Sodium dodecyl sulfate gel electrophoresis after solubilization and reduction showed the specific labeling of a single specific protein with an apparent molecular weight of 60,000 daltons. These results indicate that GnRH agonists and antagonists bind to the same receptor.

The secretion of gonadotropins from the pituitary gland is stimulated by the hypothalamic decapeptide gonadotropin releasing hormone (GnRH). The initial event in the action of GnRH is its recognition by specific binding sites (receptors) at the surface of gonadotrope cells. Studies on the interaction of metabolically stable GnRH analogs with pituitary membrane preparations or pituitary cells have indicated the presence of a single class of high affinity binding sites for both agonists and antagonists of GnRH (1-7). However, studies utilizing tritiated native GnRH have indicated both high affinity and low affinity binding sites (8). We have demonstrated that the binding of GnRH agonists and antagonists to pituitary receptors is affected differently by cations, sulfhydryl blocking reagents and lectins and by pretreatment of membranes with phospholipases, proteolytic and glycosidic enzymes (9-11).

Recently, we have identified a specific GnRH receptor protein from pituitary membrane preparations, with an apparent molecular weight of 60,000

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daltons, using an ^{125}I-labeled photoreactive GnRH agonist derivative (12). Since GnRH agonists and antagonists bind differently to the GnRH receptor, it is of interest to identify the receptors using a GnRH antagonist. In the present study, we have prepared and characterized a photoaffinity labeled GnRH antagonist. This derivative, [D-pGlu^1,D-Phe^2,D-Trp^3,D-Lys^6-(azidobenzoyl)]-GnRH, has been employed for covalent labeling of pituitary GnRH receptors.

MATERIALS AND METHODS

Materials

[D-pGlu^1,D-Phe^2,D-Trp^3,D-Lys^6]GnRH (GnRH antagonist) was kindly supplied by Dr. J.M. Stewart, Denver, Colorado. [D-Ser(t-Bu)^6,des- Gly^{10}-ethylamide] GnRH (Buserelin) was provided by Dr. J. Sandow, Hoechst, Frankfurt. (4-Azidobenzoyl)-N-hydroxysuccinimide was purchased from Pierce.

Synthesis of photoaffinity antagonist

[D-pGlu^1,D-Phe^2,D-Trp^3- D-Lys^6(N^{ε}-azidobenzoyl)]GnRH (photoaffinity antagonist) was prepared by reaction of [D-pGlu^1,D-Phe^2,D-Trp^3,D-Lys^6]GnRH with 2 molar equivalents of (4-azidobenzoyl)-N-hydroxysuccinimide in methanol-dimethylformamide in the presence of 1.2 equivalents of triethylamine. After standing at 24°C for 3 h, protected from light, the product was precipitated by the addition of ether and washed 3 times with ethyl acetate in order to remove unreacted (4-azidobenzoyl)-N-hydroxysuccinimide. Thin-layer chromatography (silica gel) revealed R_f values of 0.67 in BuOH:AcOH:H_2O:ethylacetate (4:1:1:1, by vol.) and 0.34 in acetonitrile:H_2O (9:1, by vol.).

Iodination and pituitary membrane preparations

Buserelin and the photoaffinity antagonist were iodinated by the lactoperoxidase method (9,12-14); specific activity of the labeled peptides (assessed by self-displacement assay) was approximately 1.0 mCi/µg. Pituitary membranes were prepared from 25 to 28 day-old Wistar derived female rats as described previously (9). Briefly, the glands were homogenized gently with a Dounce homogenizer at 4°C in assay buffer (10 mM Tris.HCl, pH-7.4, 0.1% bovine serum albumin, BSA) containing 1 mM dithiothreitol and centrifuged for 10 min at 1000xg. The supernatant was then centrifuged for 20 min at 20,000xg. The pellet was resuspended in assay buffer, centrifuged at 20,000xg for 20 min and finally suspended in assay buffer.

Binding assay

The labeled Buserelin (40,000 cpm) was incubated with 10 to 15 µg protein of pituitary membranes in a total volume of 0.5 ml assay buffer for 90 min at 4°C (equilibrium conditions). The binding was measured by filtration under vacuum through Whatman GF/C filters. Specific binding represents the bound radioactivity which can be competed for by 10^{-6}M unlabeled Buserelin.

Bioassay

Pituitaries derived from 12-day-old female rats were incubated in Krebs Ringer Bicarbonate containing 1 ng/ml GnRH, 10 ng/ml photoaffinity antagonist, or a combination of both peptides. The incubation procedure has been described in detail elsewhere (15). At the end of the incubation period, ali-

quots of the medium were analyzed by radioimmunoassay for LH content 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-1 reference preparation.

Photolysis and SDS-polyacrylamide gel electrophoresis

Pituitary membranes (0.6 mg) were incubated with ^{125}I-labeled photoaffinity antagonist (500,000 cpm) in the absence or presence of various concentrations of [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]GnRH or Buserelin in 1.0 ml assay buffer at 4°C in the dark. After 90 min, the membranes were photolysed (7 min at 4°C) with a 200-Watt mercury lamp at a distance of 10 cm. The membranes were washed with assay buffer by centrifugation and the pellet was boiled in 1% SDS/10 mM dithiothreitol. Aliquots were prepared and analyzed in 7.5% slab gel. After staining with Coomassie blue and destaining, the gels were dried for autoradiography.

RESULTS

[D-pGlu^1,D-Phe^2,D-Trp^3,D-Lys^6(N$^\varepsilon$-azidobenzoyl)]GnRH (photoaffinity antagonist) was prepared by chemical modification of the epsilon amino group in position 6 of [D-pGlu^1,D-Phe^2,D-Trp^3,D-Lys^6]GnRH (a GnRH antagonist) with the N-hydroxysuccinimide ester of 4-azidobenzoic acid. This antagonist was selected as the starting material for derivatization since a) substitution of the D-amino acid in the sixth position of GnRH results in a more potent and metabolically stable derivative (16), and b) the epsilon amino group of lysine serves as a spacer for substitution reactions and thus the GnRH conformation is less likely to be disturbed. The analog appeared homogeneous when analyzed by thin-layer chromatography and gave a negative test with ninhydrin reagent, indicating the absence of free amino groups. The photoreactivity of this analog was established by its spectral changes when irradiated with light (Fig. 1), and revealed spectral properties identical to those of azidobenzoyl derivatives (14,17).

The inhibition of binding of ^{125}I-labeled Buserelin to pituitary membrane preparations by the photoaffinity antagonist and by GnRH is shown in Fig. 2. Their apparent IC_{50} values (the concentration of unlabeled ligand at which the specific binding of labeled ligand is displaced by 50%) were 2 nM and 10 nM, respectively. The binding of ^{125}I-labeled photoaffinity antagonist was inhibited by unlabeled photoaffinity antagonist with an apparent IC_{50} value of 2.5 nM (not shown). Thus, the photoaffinity antagonist derivative binds to the

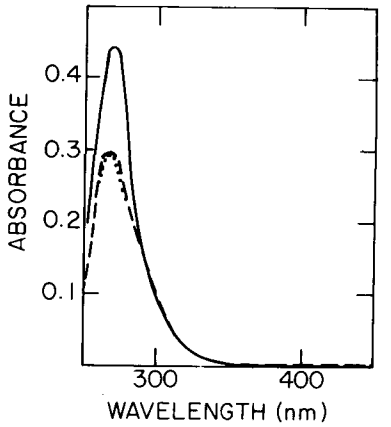

Fig. 1. The ultraviolet absorption spectra of $4x10^{-5}$M GnRH (---) and of $3x10^{-5}$M photoaffinity antagonist before (——) and after photolysis (....) in 0.1 M acetic acid.

GnRH receptor with higher apparent affinity than GnRH itself. This may be partly due to the greater resistance of this derivative to peptidase activity compared to native GnRH.

The biological activity of GnRH and the photoaffinity antagonist were assayed *in vitro* in whole pituitaries. Their effect on LH release is shown in Fig. 3. GnRH at 1 ng/ml elicited 5.4 µg/ml LH release, whereas the photoaffinity antagonist at 10 ng/ml caused only 1.0 µg/ml LH release (according to

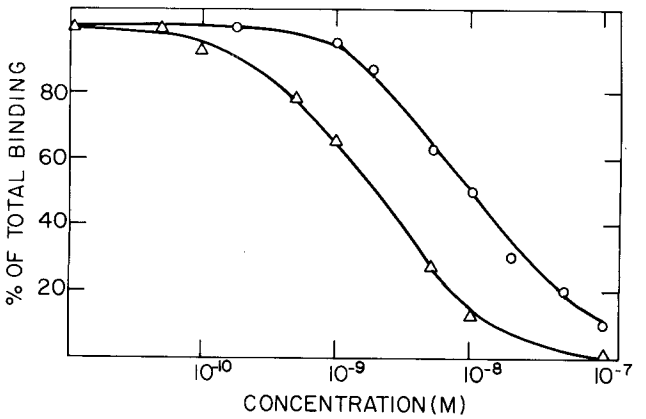

Fig. 2. Competition of binding of ^{125}I-labeled Buserelin to pituitary membranes by photoaffinity antagonist (Δ---Δ) and by GnRH (o---o). The radioactive Buserelin (30,000 cpm) was incubated with different concentrations of the tested compounds for 90 min at 4°C in a final volume of 0.5 ml containing pituitary membranes (10 to 15 µg of protein/ml) and the binding was measured as described in Materials and Methods.

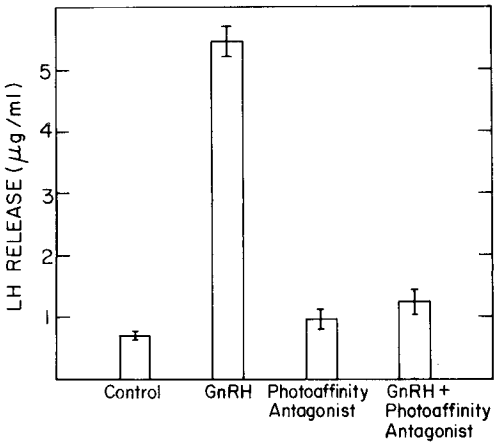

Fig. 3. Effect of GnRH (1 ng/ml), photoaffinity antagonist (10 ng/ml) and a combination of both peptides (GnRH, 1 ng/ml; photoaffinity antagonist 10 ng/ml), on LH release from pituitaries of immature female rats. The pituitaries were incubated as described in Materials and Methods. Each bar represents mean ± S.E. of 12 determinations in two separate experiments.

Student's t test, significantly not different from basal release, 0.75 μg/ml). In addition, the analog was able to inhibit GnRH-stimulated LH release. Thus, the photoaffinity derivative exhibits antagonistic activity.

Irradiation of the ^{125}I-labeled photoaffinity antagonist after pre-incubation (90 min at 4°C) with pituitary membranes, resulted (Fig. 4) in the identification of a single specific band with an apparent molecular weight of 60,000 daltons.

DISCUSSION

Aryl azide derivatives are useful tools for identification of membrane components comprising binding sites for hormones, e.g. insulin receptors (17,18), lutropin receptors (19), corticotropin receptors (20) and chemotactic receptors (21). The advantage of photo-generated nitrene intermediates is that they are highly reactive and are capable of insertion into all protein amino acid side chains.

The results presented have shown that introduction of an azidobenzoyl group into the epsilon amino side chain of the D-Lys residue of [D-pGlu^1,D-Phe^2,D-Trp^3,D-Lys^6]GnRH, results in a photoaffinity antagonist

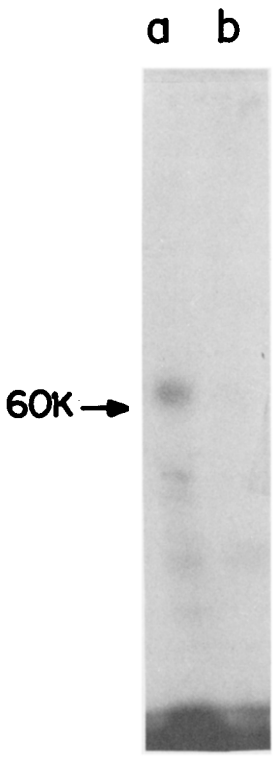

Fig. 4. Radioautograph of pituitary membrane preparations labeled with ^{125}I-labeled photoaffinity antagonist (2 x 10^{-10}M) in the absence (a) and presence of 10^{-7}M [D-pGlu^1, D-Phe^2,D-Trp^{3,6}]GnRH (b).

(Fig. 3). Photoaffinity labeling of pituitary GnRH receptor with the iodinated photoaffinity GnRH antagonist, resulted in the identification of a single specific band with an apparent molecular weight of 60,000 daltons. To further evaluate the specificity of the photolabeling the following lines of evidence have been obtained: (a) Various concentrations of unlabeled Buserelin or [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]GnRH inhibited the photolabeling of these bands in a dose-responsive manner (not shown); and (b) Thyrotropin releasing hormone at high concentrations (up to 10^{-7}M), did not affect the labeling (not shown).

Recently, we have shown that photoaffinity labeling of GnRH receptors, using a photoaffinity labeled GnRH agonist, results in the identification of a single specific band with an apparent molecular weight of 60K daltons (12). The 60K dalton band probably represents the GnRH binding sites because physiological alterations in pituitary GnRH receptor content during the rat est-

rous cycle are accompanied by similar changes in the radioactivity incorporated into this band (22). Thus, both photoaffinity labeled GnRH agonist and antagonist labeled an identical specific band of 60K daltons. This is consistent with our binding studies (9) which indicated that GnRH agonists and antagonists can interact with the same receptor. In addition, the conversion of a GnRH antagonist to an agonist by bridging two molecules, suggests that GnRH agonists and antagonists bind to the same receptor (23). However, we have also demonstrated that the binding of GnRH agonists and antagonists differs with respect to the effects of ions, lectins and by pretreatment of membranes with phospholipases, proteolytic and glycosidic enzymes (9-11). These differences in the binding characteristics may be related to charge differences in the analogs used or to possible conformational changes or steric-allosteric effects of the receptor or the hormone.

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