PHOTO-AFFINITY INACTIVATION OF GONADOTROPIN RELEASING HORMONE RECEPTORS

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1. Introduction

The secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary gland is stimulated by the hypothalamic decapeptide, gonadotropin releasing hormone (GnRH, pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly-NH_2). The first step in GnRH action is its recognition by specific binding sites (receptors) at the surface of gonadotrope cells [1]. Studies using iodinated or tritiated native hormone and pituitary membrane preparations or pituitary cells as receptor sources revealed low affinity binding sites [2–4], probably due to GnRH degradation [5,6]. Studies [7–10] which utilized binding of ^{125}I-labeled, metabolically stable GnRH analogs to pituitary plasma membrane and cultured rat pituitary cells indicated the presence of a single class of high affinity binding sites.

We have synthesized a rhodamine-labeled analog of GnRH [11] for the microscopic visualization and localization of GnRH receptors in cultured rat pituitary cells. In order to identify the membrane components comprising these GnRH binding sites, I have synthesized a photo-affinity GnRH derivative, [D-Lys^6-N^ϵ-azidobenzoyl]-GnRH. This peptide retains high affinity binding to the GnRH receptors and after photoactivation it is bound covalently to pituitary membrane preparations.

2. Materials and methods

2.1. *Synthesis of [D-Lys^6-N$^\epsilon$-azidobenzoyl]-GnRH*

pGlu–His–Trp–Ser–Tyr–D-Lys (N^ϵ-azidobenzoyl)–Leu–Arg–Pro–Gly-NH_2 was prepared by reaction of [D-Lys^6]-GnRH (0.6 mg) (Peninsula) with 2 molar equivalents of (4-azidobenzoyl)-*N*-hydroxysuccinimide (Pierce) in methanol 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 an R_F of 0.75 in BuOH:AcOH:H_2O:etylacetate (1:1:1:1, by vol.).

2.2. *Iodination and pituitary membrane preparation*

^{125}I-Labeled [D-Ser (*t*-Bu)^6, des-Gly^{10}, ethylamide]-GnRH (buserelin, Hoechst) was iodinated by the lactoperoxidase method, applied to a Sephadex G-25 column (4 × 35 cm) pre-equilibrated and eluted with 0.01 M acetic acid [12]. The specific activity of the labeled peptide was 1500 μCi/μg. Pituitary membranes were prepared from Wistar-derived female rats according to [4] with modifications. Briefly, the glands were homogenized gently with a Dounce homogenizer at 4°C in 1 mM $NaHCO_3$ containing 1 mM dithiothreitol and centrifuged for 10 min at 1000 × *g*. The supernatant was then centrifuged for 20 min at 20 000 × *g*. The pellet was resuspended in assay buffer (10 mM Tris–HCl (pH 7.4) containing 0.1% bovine serum albumin, (BSA)) centrifuged at 20 000 × *g* for 20 min and finally suspended in assay buffer.

2.3. *Binding assay*

The labeled peptide (40 000 cpm) was incubated with 200–300 μg protein of pituitary membranes in a total volume of 0.5 ml assay buffer for 90 min at 4°C. The homogenate was then filtered under vacuum through Whatman GF/C filters, presoaked in 0.1% BSA, washed with 10 ml ice-cold incubation buffer and the filters counted in a γ-counter. Specific binding

represents the bound radioactivity which can be displaced by simultaneous or prior addition of 10^{-6} M unlabeled buserelin. Each value is the mean of duplicate incubations, which varied by <7%.

2.4. *Photolysis of [D-Lys⁶-N$^{\epsilon}$-azidobenzoyl]-GnRH*

Pituitary membranes were incubated with 10^{-7} M [D-Lys⁶-*N*$^{\epsilon}$-azidobenzoyl]-GnRH or buserelin (as a control) for 90 min at 4°C protected from light. At the end of the incubation period the mixtures, in 5 ml Petri dishes, were photolyzed for 6 min at 4°C with a mercury lamp at 15 cm. After irradiation the membranes were washed and incubated (24°C) in 3 changes of 10 mM Tris–HCl containing 0.1% BSA over 1 h. To study the effect of light on spectral changes, the GnRH derivative was directly photolyzed in quartz cells, under the above conditions.

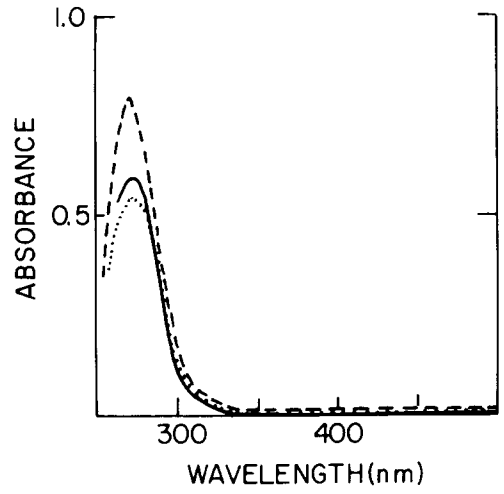

Fig.1. The ultraviolet absorption spectra of 10^{-4} M GnRH (——) and of 5×10^{-5} M [D-Lys⁶-*N*$^{\epsilon}$-azidobenzoyl]-GnRH before (– – –) and after photolysis (. . .) in 0.1 M acetic acid.

3. Results and discussion

[D-Lys⁶-*N*$^{\epsilon}$-azidobenzoyl]-GnRH was prepared by chemical modification of the ϵ-amino group in position 6 of [D-Lys⁶]-GnRH with the *N*-hydroxysuccinimide ester of 4-azidobenzoic acid, in a similar way to our preparation of the fluorescent derivative of GnRH, [D-Lys⁶-*N*$^{\epsilon}$-tetramethylrhodamine]-GnRH [11]. [D-Lys⁶]-GnRH was selected as the starting material for derivatization since:

(i) Substitution of D-amino acids in position 6 of GnRH results in more potent and metabolically stable derivatives [5,6];
(ii) The ϵ-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 [13].

The inhibition of binding of ^{125}I-labeled buserelin to pituitary membrane preparations by [D-Lys⁶-*N*$^{\epsilon}$-azidobenzoyl]-GnRH and GnRH is shown in fig.2. The app. IC_{50}-values (the concentration of unlabeled ligand at which the specific binding of labeled ligand is displaced by 50%) were found to be 0.4 nM and 10 nM for [D-Lys⁶-*N*$^{\epsilon}$-azidobenzoyl]-GnRH and GnRH, respectively. The photo-affinity derivative retains higher affinity than our fluorescent derivative [D-Lys⁶-*N*$^{\epsilon}$-tetramethylrhodamine]-GnRH (3 nM, [11]), since azidobenzoyl is a less bulky group than rhodamine. However, both bind to the GnRH receptor with higher affinity than GnRH itself probably due to the increased peptidase resistance of these derivatives.

Photoactivation of 10^{-7} M [D-Lys⁶-*N*$^{\epsilon}$-azidobenzoyl]-GnRH after preincubation (90 min at 4°C) with pituitary membranes (fig.3) resulted in a decrease of ~80% of the specific binding of ^{125}I-labeled buserelin.

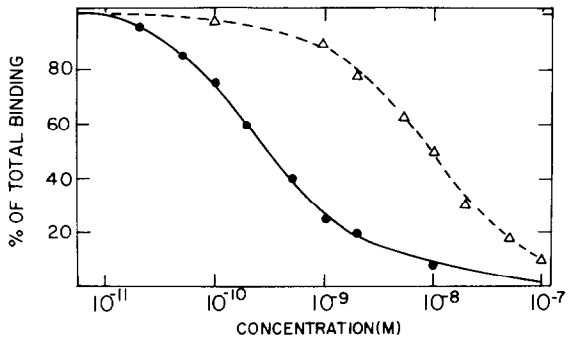

Fig.2. Competition in binding of ^{125}I-labeled buserelin to pituitary plasma membrane by GnRH (△– – –△) and [D-Lys⁶-*N*$^{\epsilon}$-azidobenzoyl]-GnRH (●——●). The radioactive buserelin (40 000 cpm) was incubated with different concentrations of the tested compound for 90 min at 4°C in a final volume of 0.5 ml containing pituitary membranes (0.2–0.3 mg protein/ml) and the binding was measured as in section 2.

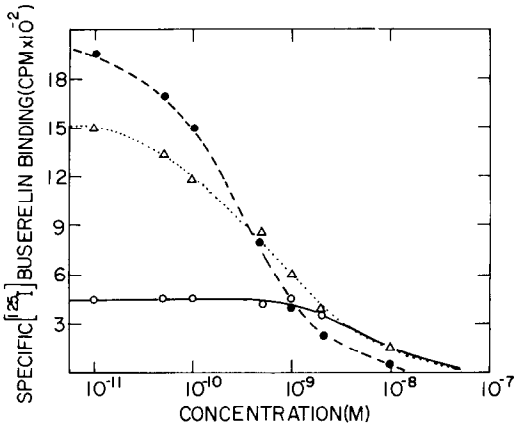

Fig.3. Competition of binding of ^{125}I-labeled buserelin by unlabeled buserelin to control pituitary membranes (●——●), pituitary membranes photolyzed in the presence of 10^{-7} M buserelin (△. . .△) or 10^{-7} M [D-Lys^6-N^{ϵ}-azidobenzoyl]-GnRH (○——○). The conditions are as in section 2 and fig.2.

Irradiation in the presence of 10^{-7} M buserelin resulted in a small reduction of the total specific binding. These results indicate that the photoaffinity analog is bound covalently to the pituitary membrane preparation after photolysis. Although the occupancy of the GnRH receptors at 10^{-7} M [D-Lys^6-N^{ϵ}-azidobenzoyl]-GnRH was ~100% (fig.2), irradiation at this concentration abolished only 80% of the total receptors (fig.3). This is consistent with the observation [14] that the yield of photo-affinity labeling is very low.

The data indicate that the photo-affinity derivative of GnRH can interact, with high affinity, with the GnRH receptors and that after photoactivation it is bound covalently to pituitary membranes. This analog can be easily iodinated and utilized for identification of the membrane components comprising the GnRH binding sites. 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, as well as other components of the membrane.

Acknowledgements

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