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Mapping of Gonadotropin-Releasing Hormone Receptor Binding Site†

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ABSTRACT: On the basis of the spatial conformation of gonadotropin-releasing hormone (GnRH), we have predicted that aromatic amino acids and at least one carboxyl group are involved in the recognition site of the receptor. Therefore, various specific reagents were examined for their ability to interfere with the binding of GnRH to its receptor. Pretreatment of pituitary membrane preparations with sodium periodate decreased the specific binding in a dose-dependent manner (IC_{50} = 0.5 mM) due to a decrease in receptor affinity. This indicated the presence of a sugar moiety in the binding site. Tryptophan is another constituent that participates in the GnRH binding site, as pretreatment of pituitary membranes with 2-methoxy-5-nitrobenzyl bromide inhibited the binding (IC_{50} = 0.22 mM) by decreasing receptor affinity. In addition, the native hormone conferred on the binding site a protective effect against inactivation by 2-methoxy-5-nitrobenzyl bromide. Pretreatment of membranes with *p*-diazobenzenesulfonic acid also inhibited the binding of ^{125}I-Buserelin (IC_{50} = 0.1 mM), indicating the presence of tyrosine within or near the binding site. Pretreatment of pituitary membrane preparations with dithiothreitol also inhibited the binding due to a decrease in the binding affinity, which was accompanied by an increase in receptor number. These data suggest that there are disulfide bonds within or near the binding region. Treatment with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and glycine ethyl ester also prevented binding in a dose-dependent manner and implies that free carboxylic groups are involved in the binding site. Since divalent cations were more potent in inhibiting the specific binding in comparison with monovalent cations and since the order of potency within the divalent cations was identical with their association constants to dicarboxylic compounds, it is suggested that there are at least two carboxylic groups that participate in the binding of the hormone. According to these findings, we propose a model describing the interaction involved in the formation of the hormone–receptor complex.

The secretion of gonadotropins from the pituitary gland is stimulated by the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH).[1] The first step in GnRH action is its interaction with specific receptors at the surface of gonadotrope cells (Conn et al., 1981). Photoaffinity labeling of the GnRH receptors of rat pituitary membrane preparations resulted in the identification of a single specific band with an apparent M_r of 60K (Hazum, 1981a,b; Hazum & Keinan, 1983). Treatment of rat pituitary membranes with trypsin, chymotrypsin, and neuraminidase resulted in a decrease of the specific binding of iodinated GnRH agonist and antagonist (Hazum, 1981c, 1982). These results indicated that the GnRH receptor is a glycoprotein. Moreover, preincubation of membrane preparations with wheat germ agglutinin, a specific lectin for sialic acid and *N*-acetylglucosamine, caused a marked inhibition of GnRH agonist and antagonist binding, suggesting that sialic acid is an integral component of the GnRH receptor, essential for binding (Hazum, 1982). Digestion of pituitary membranes with various phospholipases reduced the specific binding of both GnRH agonist and antagonist, implicating membrane phospholipid involvement in the hormone–receptor interaction (Hazum et al., 1982). In this study we have used specific chemical reagents to analyze the components comprising the binding site, essential for the recognition of the hormone.

Materials and Methods

Materials

All reagents used were purchased from the usual commercial sources.

Methods

Iodination and Pituitary Membrane Preparations. [D-Ser(Bu^t)^6,des-Gly^{10},ethylamide]GnRH (Buserelin, GnRH agonist provided by Dr. J. Sandow, Hoechst) was iodinated by the lactoperoxidase method (Sandow & Konig, 1979). Specific activity of the labeled peptide was approximately 1.0 mCi/μg, as measured by self-displacement in the pituitary radioligand receptor assay. Pituitary membranes were prepared from 25 to 28 day old Wistar-derived female rats according to Heber & Odell (1978), with modification. Briefly, the glands were homogenized gently with a tight Dounce homogenizer at 4 °C in assay buffer [10 mM Tris-HCl, pH

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[1] Abbreviations: GnRH, gonadotropin-releasing hormone; Buserelin, [D-Ser(Bu^t)^6,des-Gly^{10},ethylamide]GnRH; DTT, dithiothreitol; GEE, glycine ethyl ester; DCI, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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7.4, 0.1% bovine serum albumin (BSA)] and centrifuged for 10 min at 1000*g*. The supernatant was then centrifuged for 20 min at 20000*g*. The pellet was resuspended in assay buffer, centrifuged at 20000*g* for 20 min, and finally suspended in assay buffer.

Binding Assay. The labeled Buserelin (40 000 cpm) was incubated with pituitary membranes (50–100 μg of protein) in a total volume of 0.5 mL of 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 in the presence of 10^{-7} M unlabeled Buserelin subtracted from the total bound radioactivity. Each point is the mean of triplicate incubations from at least two separate experiments, which varied by less than 10%. In cases when the variations were more than 10%, the mean ± standard error is included.

Treatment with Different Reagents. Pituitary membrane preparations were incubated with different concentrations of a reagent for 30 or 60 min at room temperature in 1 mL of 10 mM phosphate buffer, pH 7.4. At the end of the incubation period the membranes were precipitated and washed twice. Each wash consisted of a 15-min incubation with 20 mL of 10 mM Tris-HCl, pH 7.4, containing 0.1% BSA and centrifugation (20 min at 20000*g*). The membranes were resuspended in assay buffer, and the binding was assayed.

p-Diazobenzenesulfonic acid was prepared immediately before use as follows: *p*-sulfanilic acid (10 μmol) in 25 μL of cold 2 N HCl was diazotized by the addition of sodium nitrite (10 μmol) in cold water (25 μL, 4 °C). After this was allowed to stand for 8 min at 4 °C, 75 μmol of $NaHCO_3$ dissolved in 150 μL ice-cold water (pH 8.5) was added. This reaction mixture in the appropriate concentration was immediately reacted with pituitary membranes. The colorless solution turned orange-brown within a few minutes; the reaction was allowed to proceed for an additional 1 h.

Competition Binding Experiments and Scatchard Analysis. Pituitary membrane preparations were treated with 0.5 mM 2-methoxy-5-nitrobenzyl bromide (60 min, 24 °C), 10 mM dithiothreitol (30 min, 24 °C), or 0.5 mM sodium periodate (60 min, 24 °C) in 1 mL of 10 mM phosphate buffer, pH 7.4. Control (untreated) membranes were incubated with 1 mL of 10 mM phosphate buffer, pH 7.4, for 30 or 60 min at 24 °C. Following extensive washing (as described above), competition binding experiments with untreated and treated membranes were carried out by incubating radioactive Buserelin (40 000 cpm) and various concentrations of unlabeled Buserelin (10^{-12}–10^{-7} M) in 0.5 mL of assay buffer. After 90 min at 4 °C, binding was plotted according to Scatchard (1949) and analyzed statistically by the method of Wilkinson (1961).

Protection Experiment. Pituitary membranes were suspended in 2 mL of 10 mM phosphate buffer, pH 7.4. To half of the membranes 10^{-6} M GnRH was added, and both were incubated for 90 min at 4 °C. At the end of the incubation (time 0), 200 μL of each sample was centrifuged (3 min, 20000*g*). The residual samples were incubated with 0.5 mM 2-methoxy-5-nitrobenzyl bromide; at 5, 10, 20, and 30 min, samples of 200 μL were centrifuged. The membrane pellets were extensively washed with assay buffer and binding was assayed.

Results

The ability of various reagents to interfere with the specific binding of iodinated Buserelin to pituitary membrane preparations was tested. Sodium periodate is known to break the bond between vicinal hydroxyl groups of a sugar moiety.

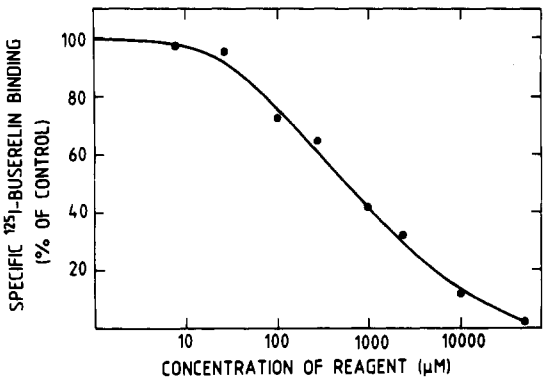

FIGURE 1: Inhibition of the binding of ^{125}I-labeled Buserelin to pituitary membrane preparations by sodium periodate. The membranes were washed in 10 mM phosphate buffer (PB) and incubated with different concentrations of sodium periodate (0.01–10 mM) in PB, for 1 h at room temperature. The membranes were washed twice with 10 mM Tris and 0.1% BSA (assay buffer), and binding was assayed as described under Materials and Methods.

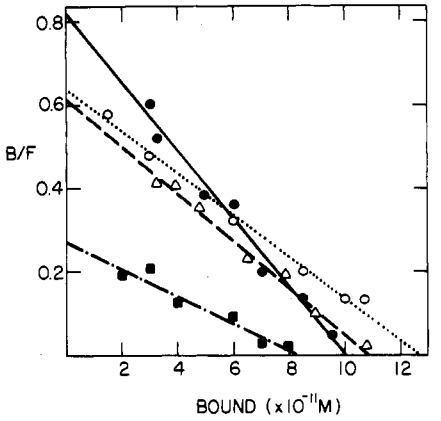

FIGURE 2: Effect of reagent treatment on receptor binding parameters. Scatchard curves were plotted from the competition binding experiment of ^{125}I-Buserelin with various concentrations of unlabeled Buserelin, as described under Materials and Methods. Control membranes (●) and membranes pretreated with 0.5 mM sodium periodate (△), with 0.5 mM 2-methoxy-5-nitrobenzyl bromide (■), or with 10 mM dithiothreitol (○). Scatchard plots were obtained from a representative experiment conducted in triplicate.

Table I: IC_{50} Values for the Inhibitory Effect of Various Reagents on ^{125}I-Buserelin Binding[a]

reagent	IC_{50} (mM)
sodium periodate	0.5
2-methoxy-5-nitrobenzyl bromide	0.22
p-diazobenzenesulfonic acid	0.1
dithiothreitol	14
1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide + glycine ethyl ester	25

[a] IC_{50} is the concentration of reagent that inhibits the specific binding of ^{125}I-Buserelin to pituitary membrane preparations by 50%.

Pretreatment of pituitary membrane preparations with this reagent showed a dose-dependent decrease in the specific binding of ^{125}I-Buserelin (Figure 1), with an IC_{50} value of 0.5 mM (Table I). The kinetics of binding of iodinated Buserelin to sodium periodate (0.5 mM) treated membranes, in the presence of different concentrations of unlabeled Buserelin, was next examined. Scatchard analysis (Figure 2) of the inhibition binding curve revealed a decrease in the affinity of the receptor from 0.12 nM in control membranes to 0.18 nM in pretreated membranes, accompanied by a slight increase in the number of receptors (Table II). The binding affinity of control membranes is higher than the affinity detected in

Table II: Effect of Reagent Treatment on Binding Affinity and Number of GnRH Receptors[a]

reagent	K_d (nM)	B_{max} (fmol/pituitary)
control	0.12 ± 0.01	165 ± 4
sodium periodate (0.5 mM)	0.18 ± 0.01	180 ± 3
2-methoxy-5-nitrobenzyl bromide (0.5 mM)	0.29 ± 0.04	135 ± 6
dithiothreitol (10 mM)	0.22 ± 0.02	210 ± 7

[a] B_{max} and K_d values (mean ± SEM) were calculated from the representative experiment in Figure 2 according to Wilkinson (1961).

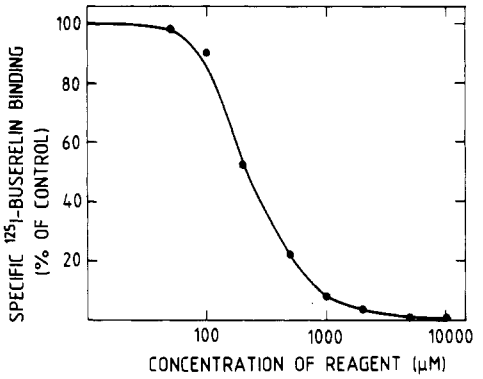

FIGURE 3: Inhibition of the binding of ^{125}I-labeled Buserelin to pituitary membrane preparations by 2-methoxy-5-nitrobenzyl bromide. The membranes were washed and incubated in 10 mM phosphate buffer with different concentrations of 2-methoxy-5-nitrobenzyl bromide (0.1–10 mM) dissolved in dimethylformamide. Experimental details are as in the legend to Figure 1.

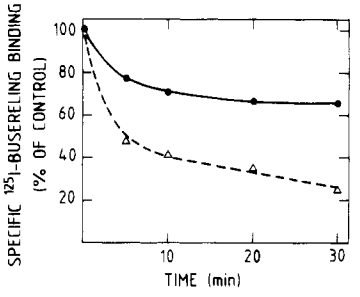

FIGURE 4: Time course of inactivation of GnRH receptors by 2-methoxy-5-nitrobenzyl bromide of control membranes (△) and GnRH-protected membranes (●). Inactivation was performed as described under Materials and Methods.

our previous studies (Hazum, 1981c) because in the present study dithiothreitol and sodium bicarbonate were not present during the homogenization of pituitary glands.

Figure 3 shows the effect of various concentrations of 2-methoxy-5-nitrobenzyl bromide on the specific binding of ^{125}I-Buserelin to pituitary membrane preparations (IC_{50} = 0.22 mM, Table I). This reagent is known to specifically modify the indole residue of the amino acid tryptophan (Means & Feeney, 1971), suggesting that this amino acid participates in the binding site. Scatchard analysis (Figure 2) of competition binding experiments to membranes treated with 0.5 mM 2-methoxy-5-nitrobenzyl bromide revealed a marked decrease in receptor affinity (0.29 nM) as compared to control values (0.12 nM), accompanied by a small decrease in the number of receptors (Table II). To confirm that the inhibition of binding is a result of a direct modification of the binding site, the protective effect of GnRH against inactivation by one of the reagents was tested. As shown in Figure 4, the inactivation by 2-methoxy-5-nitrobenzyl bromide as a function of time indicated that the hormone-bound receptor was less accessible to the reagent than unoccupied receptor.

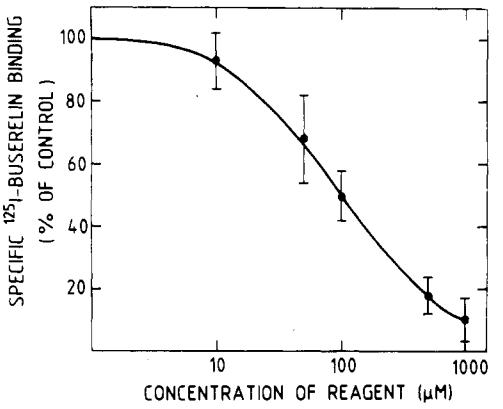

FIGURE 5: Dose–response curve for the inhibition of ^{125}I-Buserelin binding by *p*-diazobenzenesulfonic acid. *p*-Diazobenzenesulfonic acid was prepared before use as described under Materials and Methods. The reaction mixture in the appropriate concentration was immediately reacted with pituitary membranes and incubated for 1 h at room temperature. Following extensive washing, binding was assayed. Points are means ± SEM of nine determinations in three separate experiments.

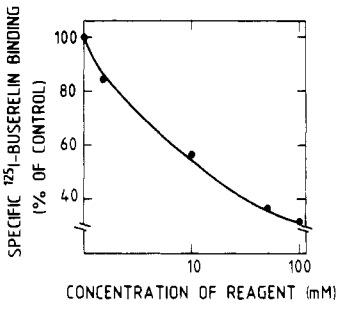

FIGURE 6: Dose-dependent inhibition of ^{125}I-Buserelin binding to pituitary membrane preparations by DTT. Membranes were washed and incubated with DTT (1–100 mM) for 30 min at room temperature. Binding was assayed in the presence of DTT as described under Materials and Methods.

The participation of tyrosine in the receptor recognition site is indicated by the observation that *p*-diazobenzenesulfonic acid, which is known to react with the imidazole ring of histidine and the phenol ring of tyrosine (Means & Feeney, 1971), inhibited the binding of ^{125}I-Buserelin (Figure 5; IC_{50} = 0.1 mM, Table I). However, it seems that the presence of histidine in the binding site can be excluded, because treatment of pituitary membrane preparations with iodoacetamide (in concentrations from 0.1 to 100 mM), which reacts with histidine and sulfhydryl groups, did not affect the binding (data not shown). The absence of free sulfhydryl groups in the binding site is also evident from the fact that pretreatment of the membranes with *N*-ethylmaleimide (0.1–100 mM) had no effect on the binding.

When pituitary membrane preparations were exposed to different concentrations of dithiothreitol (DTT) and the binding assay was conducted in the presence of DTT or subsequent to extensive washing, there was a similar decrease in the specific binding (Figure 6; IC_{50} = 14 mM, Table I). Scatchard analysis (Figure 2) of competition binding of membranes pretreated with 10 mM DTT revealed a slight decrease in affinity, while the number of receptors increased (Table II). These findings suggest that disulfide bonds play a role in GnRH binding.

Pretreatment of pituitary membrane preparations with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (DCI), which activates carboxylic groups (Means & Feeney, 1971), had a small effect on the specific binding of ^{125}I-Buserelin,

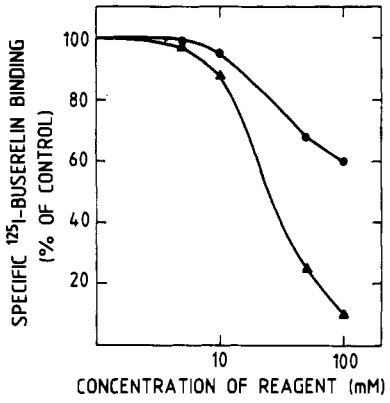

FIGURE 7: Dose–response curve for the inhibition of ^{125}I-labeled Buserelin binding to pituitary membranes by DCI (5–100 mM) (●) and DCI + GEE (5–100 mM) (▲). The membranes suspended in 10 mM phosphate buffer were allowed to react with different concentrations of the reagents for 1 h at room temperature. The membranes were extensively washed in 10 mM Tris and 0.1% BSA, and subsequently, binding was assayed as described under Materials and Methods.

Table III: IC_{50} Values for the Inhibitory Effect of Monovalent and Divalent Cations on ^{125}I-Buserelin Binding[a]

cation	IC_{50} (mM)	cation	IC_{50} (mM)
Cs^+	25	Mg^{2+}	0.9
Na^+	24	Mn^{2+}	0.4
K^+	20	Zn^{2+}	0.15
Ca^{2+}	1.0	Cu^{2+}	0.05

[a] IC_{50} is the concentration of cation that inhibits the specific binding of ^{125}I-Buserelin to pituitary membrane preparations by 50%.

whereas pretreatment with DCI and glycine ethyl ester (GEE), which form a stable amide bond, blocked the binding site more effectively (Figure 7; IC_{50} = 25 mM, Table I). These data indicate that there is a free carboxyl group involved in GnRH binding that can be attributed to a sialic acid residue or to a glutamic or aspartic acid side chain. Pretreatment of the membranes with the *N*-hydroxysuccinimide ester of acetic acid (0.01–100 mM) did not affect the binding, indicating the absence of free amino groups in the binding site (data not shown).

The inhibitory effect of different cations on the binding of ^{125}I-Buserelin to pituitary membrane preparations was next examined (Figures 8 and 9 and data not shown for $CaCl_2$ and $MgCl_2$). Monovalent cations require higher concentrations than divalent cations to inhibit the binding (Table III). The degree of inhibition of binding by the divalent cations was in the order Cu > Zn > Mn > Mg = Ca; this is parallel to the association constants of these cations to compounds with two carboxylic groups (Martell & Sillen, 1964). These results suggest that at least two carboxylic groups are involved in the recognition site of the receptor.

Discussion

We have used several reagents to identify the functional entities of GnRH receptor that are involved in the binding of the hormone. The present findings suggest that the binding site includes a sugar moiety because pretreatment of pituitary membranes with sodium periodate abolished the specific binding. A similar decrease in the specific binding has previously been shown in neuraminidase-treated membrane (Hazum, 1982); this enzyme removes the terminal sialic acid residue attached to membrane glycoprotein structures. These results indicate that a sugar moiety, probably sialic acid, participates in the binding of the hormone to the GnRH receptor of the pituitary.

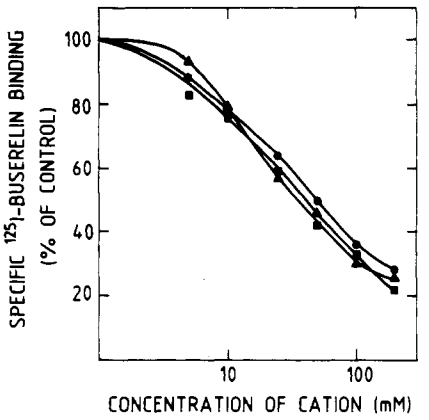

FIGURE 8: Inhibition of the binding of ^{125}I-Buserelin to pituitary membrane preparations by monovalent cations. The radioactive Buserelin (50 000 cpm) was incubated with various concentrations (5–200 mM) of the monovalent cations [(●) NaCl; (▲) KCl; (■) CsCl] for 90 min at 4 °C in a final volume of 0.5 mL containing pituitary membranes (50–100 μg of protein/mL). The binding was assayed as described under Materials and Methods.

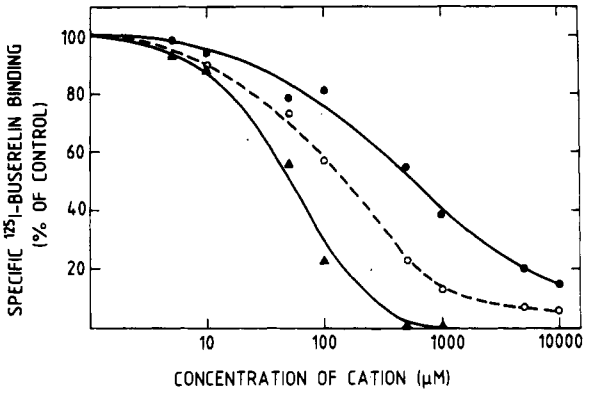

FIGURE 9: Inhibition of the binding of ^{125}I-labeled Buserelin to pituitary membrane preparations by divalent cations. Experimental details as in the legend to Figure 7; (●) $MnCl_2$, (▲) $CuCl_2$, and (○) $ZnCl_2$.

Our data also indicate that the amino acids tryptophan and tyrosine are associated with the GnRH binding site, since pretreatment with 2-methoxy-5-nitrobenzyl bromide or *p*-diazobenzenesulfonic acid inhibited the binding. Supporting evidence that the impairment of GnRH binding after reagent treatment is due to a modification of the amino acid side chains involved comes from the protective effect of GnRH against inactivation by 2-methoxy-5-nitrobenzyl bromide. Free sulfhydryl groups are probably not involved in the GnRH binding site since specific alkylating reagents such as *N*-ethylmaleimide and iodoacetamide did not affect the binding. Nevertheless, treatment of pituitary membranes with DTT results in a decreased affinity while receptor content increased significantly. These findings suggest that either there are disulfide bonds within the binding site of the receptor or a reduction of disulfide bonds at a remote location from the binding site may change the conformation of the receptor, resulting in a reduction in affinity. The increased number of receptors is probably due to exposure or unmasking of receptor sites.

Pretreatment of the membranes with DCI plus GEE suggests that at least one carboxyl group is present within or near the binding site. Since the potency of the divalent cations in inhibiting the binding correlates with their association constant to dicarboxylic compounds (Martell & Sillen, 1964), the participation of two carboxyl groups in the binding site may be inferred. One of the carboxyl groups is probably associated

with sialic acid, and the other can be attributed to either aspartic or glutamic acid or to an additional sialic acid. Nevertheless, the effects of divalent cations can be related to possible conformational changes or steric–allosteric effects on the receptor (Gurwitz & Sokolovsky, 1980) or the hormone. Other possibilities include interactions with phospholipids or alteration of the hydrophobic environment at the binding site. A similar effect of cations on GnRH–receptor interactions has also been observed by other investigators (Hazum, 1981c; Perrin et al., 1983; Loumaye et al., 1984).

Gonadotropin-releasing hormone is a decapeptide with the following amino acid sequence: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. Three-dimensional analysis of the hormone in solution indicated that there is a β-turn in position 6, which brings the carboxy and the amino termini of the hormone into close proximity. It has been suggested that the side chains of histidine, tyrosine, and arginine form a packed unit that may play an active role in the hormone action. Tryptophan, however, is at a maximal distance from this unit and thus may act as an independent active entity (Shinitzky & Fridkin, 1976; Shinitzky et al., 1976). According to the spatial conformation of the native hormone, we can postulate a model for its interaction with the receptor. It is well-known that arginyl residues on proteins may serve as positively charged loci for recognition of negatively charged anions. Thus, the driving force for the formation of the hormone–receptor complex is probably an ionic interaction between the amino acid arginine in position 8, which is positively charged, and the carboxyl groups in the binding site. In addition to the ionic interaction, the hormone–receptor complex is stabilized by aromatic π–π interactions between the histidine, tryptophan, and tyrosine residues in the hormone and tyrosine and tryptophan in the receptor site. Finally, phospholipids play an apparent role in the structural integrity of the receptor since we have shown (Hazum et al., 1982) that the binding of GnRH is acutely dependent on both the exterior hydrophilic head groups and the fatty acid on the β-carbon of phospholipid.

Registry No. LH-RH, 9034-40-6; buserelin, 57982-77-1; tryptophan, 73-22-3; tyrosine, 60-18-4.

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