Sompayrac, L. M., & Danna, K. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7575.
Southern, P. J., & Berg, P. J. (1982) *J. Mol. Appl. Genet. 1*, 327.
Stenflo, J. (1976) *J. Biol. Chem. 251*, 355.
Sugo, T., Persson, V., & Stenflo, J. (1985) *J. Biol. Chem. 260*, 10453.
Suttie, J. W., Hoskins, J. A., Engelke, J., Hopfgartner, A., Ehrlich, H., Bang, N. U., Belagaje, R. M., Schoner, B., & Long, G. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 634.
von Heijne, G. (1984) *J. Mol. Biol. 173*, 243.
Yoshitake, S., Schach, B. G., Foster, D. C., Davie, E. W., & Kurachi, K. (1985) *Biochemistry 24*, 3736.
Zoller, M., & Smith, M. (1982) *Nucleic Acids Res. 10*, 6487.

Binding Properties of Solubilized Gonadotropin-Releasing Hormone Receptor: Role of Carboxylic Groups†

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ABSTRACT: The interaction of ^{125}I-buserelin, a superactive agonist of gonadotropin-releasing hormone (GnRH), with solubilized GnRH receptor was studied. The highest specific binding of ^{125}I-buserelin to solubilized GnRH receptor is evident at 4 °C, and equilibrium is reached after 2 h of incubation. The soluble receptor retained 100% of the original binding activity when kept at 4 or 22 °C for 60 min. Mono- and divalent cations inhibited, in a concentration-dependent manner, the binding of ^{125}I-buserelin to solubilized GnRH receptor. Monovalent cations require higher concentrations than divalent cations to inhibit the binding. Since the order of potency within the divalent cations was identical with that of their association constants to dicarboxylic compounds, it is suggested that there are at least two carboxylic groups of the receptor that participate in the binding of the hormone. The carboxyl groups of sialic acid residues are not absolutely required for GnRH binding since the binding of ^{125}I-buserelin to solubilized GnRH receptor was only slightly affected by pretreatment with neuraminidase and wheat germ agglutinin. The finding that polylysines stimulate luteinizing hormone (LH) release from pituitary cell cultures with the same efficacy as GnRH suggests that simple charge interactions can induce LH release. According to these results, we propose that the driving force for the formation of the hormone–receptor complex is an ionic interaction between the positively charged amino acid arginine in position 8 and the carboxyl groups in the binding site.

The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH,[1] pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is the primary regulator of the reproductive cycle. Binding of this hormone to specific receptors on pituitary gonadotrope cell membranes initiates the processes that lead to gonadotropin release. Characterization of the GnRH receptor in the pituitary has indicated that the receptor is a sialoglycoprotein with an apparent M_r of 60K (Hazum, 1981a,b, 1982; Schvartz & Hazum, 1985). Furthermore, using specific chemical reagents, we have identified two aromatic amino acid residues and two carboxylic groups in the membrane-associated GnRH receptor that are likely to be essential for the formation of the hormone–receptor complex (Keinan & Hazum, 1985).

Recently, we have succeeded in solubilizing the GnRH receptor from rat and bovine pituitary membrane preparations in an active form (Hazum et al., 1986, 1987) by using the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). In the present study, we report the characterization of the binding properties of the solubilized GnRH receptor, as well as the role of carboxylic groups of the receptor in both GnRH binding and the biological activity.

Materials and Methods

Materials

CHAPS and neuraminidase type V were purchased from Sigma, Dextran T-70 from Pharmacia, charcoal (Norit A) was from Fisher, wheat germ agglutinin (WGA) was from Miles Yeda, and Na^{125}I was from Amersham. [D-Ser(*t*-Bu)^6]des-Gly^{10}-GnRH ethylamide (buserelin) was kindly supplied by Dr. J. Sandow, Hoechst, Frankfurt. Polylysine and poly(glutamic acid) were generously supplied by I. Jakobson.

Methods

Iodination and Pituitary Membrane Preparations. [D-Ser(*t*-Bu)^6]des-Gly^{10}-GnRH ethylamide (buserelin) 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 25–28-day-old Wistar-derived female rats according to Heber and Odell (1978), with modification. Briefly, the glands were homogenized gently with a tight Dounce homogenizer at 4 °C in 10 mM Tris·HCl, pH 7.4, and

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[1] Abbreviations: GnRH, gonadotropin-releasing hormone; buserelin, [D-Ser(*t*-Bu)^6]des-Gly^{10}-GnRH ethylamide; LH, luteinizing hormone; WGA, wheat germ agglutinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; PBS, phosphate-buffered saline.

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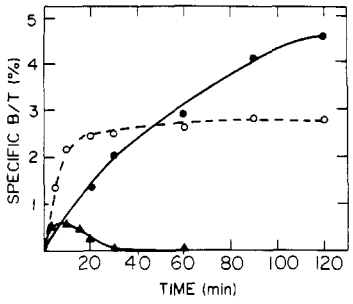

FIGURE 1: Time and temperature dependence of ^{125}I-buserelin binding to GnRH-solubilized receptor. The solubilized receptor (25 μg of protein) was incubated with ^{125}I-labeled buserelin (50 000 cpm) at 4 (●), 22 (○), or 37 °C (▲). At the indicated time intervals, the binding was terminated as described under Materials and Methods.

centrifuged for 10 min at 1000g. The supernatant was then centrifuged for 20 min at 20000g. The pellet was resuspended in 10 mM Tris·HCl buffer and centrifuged at 20000g for 20 min, and the pellet was stored at −20 °C.

Solubilization of GnRH Receptors. Pituitary membrane preparations were solubilized as described (Hazum et al., 1986), with modifications. The pellet was suspended in 10 mM Tris buffer containing 5 mM CHAPS, shaken for 60 min at 4 °C, and centrifuged (60 min at 100000g). This procedure was repeated, and the supernatants were combined and used to measure binding.

Binding Assays to Membranal and Solubilized Receptors. The binding to membranal receptors was assessed as previously described (Hazum, 1982). Briefly, the labeled buserelin (50 000 cpm) was incubated with pituitary membrane preparations (10–15 μg of protein) in a total volume of 0.5 mL of 10 mM Tris·HCl buffer containing 0.1% bovine serum albumin (BSA) for 90 min at 4 °C (equilibrium conditions). The binding was measured by filtration under vacuum through Whatman GF/C filters. The solubilized receptors (25–30 μg of protein) were incubated with the labeled buserelin (50 000 cpm) in 0.5 mL of 10 mM Tris/0.1% BSA containing 1 mM CHAPS for 2.5 h at 4 °C. The reaction was stopped by the addition of 0.3 mL of ice-cold Dextran-coated charcoal (0.5 g of Dextran T-70 and 5.0 g of activated charcoal dissolved in 1000 mL of PBS). The solutions were left on ice for 10 min and then centrifuged for 20 min at 2000g at 4 °C. The supernatants were collected and counted in a γ counter. 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%.

Biological Activity. The biological activity was assessed by the quantitation of LH released from cultured pituitary cells as described (Schvartz & Hazum, 1985). After 48 h in culture, the cells were incubated with the tested compound (4 h at 37 °C) and aliquots of the medium were taken for LH determination by radioimmunoassay using a kit supplied by the NIADDK, Rat Pituitary Hormone Program, NIH, Bethesda, MD.

RESULTS

The time course of ^{125}I-buserelin binding to solubilized GnRH receptor, at three different temperatures, is shown in Figure 1. The highest specific binding over total (B/T) is evident at 4 °C, and equilibrium is reached after 120 min of incubation. At 22 °C, maximal binding (about 60% of that observed at 4 °C) is obtained after 20 min, whereas at 37 °C there is very low specific binding. Next, the stability of soluble

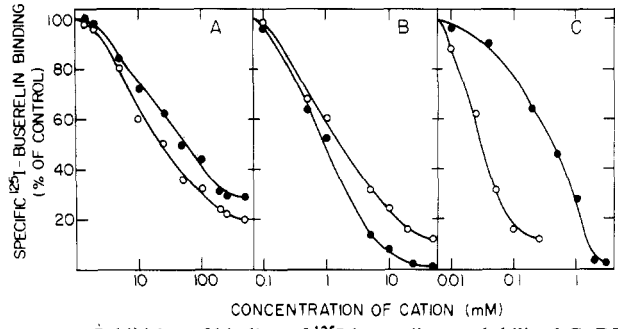

FIGURE 2: Inhibition of binding of ^{125}I-buserelin to solubilized GnRH receptor by monovalent and divalent cations. Radioactive buserelin (50 000 cpm) was incubated with increasing concentrations of the cations [(A) ●, NaCl; ○, KCl; (B) ●, $CaCl_2$; ○, $MgCl_2$; (C) ●, $MnCl_2$; ○, $CuCl_2$] for 2.5 h at 4 °C in a final volume of 0.5 mL containing solubilized GnRH receptor (25 μg of protein). The binding was assayed as described under Materials and Methods.

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

cation	IC_{50} (mM) solubilized receptor	IC_{50} (mM) membranal receptor	cation	IC_{50} (mM) solubilized receptor	IC_{50} (mM) membranal receptor
K^+	10	20	Mg^{2+}	1	0.9
Na^+	12	24	Mn^{2+}	0.4	0.4
Ca^{2+}	1	1	Cu^{2+}	0.025	0.05

[a] IC_{50} is the concentration of cation that inhibits the specific binding of ^{125}I-buserelin to solubilized or membrane-associated GnRH receptor by 50%. Data for membranal receptor are from Keinan and Hazum (1985).

GnRH receptor at 22 or 37 °C was examined. Preincubation of the solubilized receptor at 22 °C and subsequent binding assay at 4 °C did not alter the maximal specific binding (up to 60 min). In contrast, preincubation of the solubilized receptor at 37 °C caused an irreversible and progressive decrease in the specific binding (to 8% by 60 min).

The effect of different cations on the binding of ^{125}I-buserelin to solubilized GnRH receptor was next examined (Figure 2). As shown in Table I, the cations exhibited a similar inhibitory effect on ^{125}I-buserelin binding to both solubilized and membrane-associated GnRH receptor. Monovalent cations require higher concentrations than divalent cations to inhibit the binding (Table I), and the degree of inhibition of binding by the divalent cations was in the order Cu > Mn > Mg = Ca. This order is parallel to that of the association constants of these divalent cations to compounds with two carboxylic groups (Martell & Sillen, 1964), suggesting that at least two carboxylic groups are involved in the recognition site of the receptor.

To investigate whether one of the carboxyl groups is associated with a sialic acid residue, we examined the effect of neuraminidase and WGA on ^{125}I-buserelin binding. Neuraminidase treatment was conducted at 22 °C and pH 7.4, since under optimal conditions for enzyme activity (37 °C, pH 5.5), the solubilized receptor was inactivated; BSA was present during the preincubation time to prevent proteolytic activity. As shown in Table II, pretreatment with neuraminidase decreased the specific binding of ^{125}I-buserelin to both the solubilized and membrane-associated receptor. Because neuraminidase selectively cleaves exposed, terminal sialic acid residues of membrane glycoproteins, and because the solubilized receptor is less sensitive to neuraminidase treatment, these results indicate that sialic acid (*N*-acetylneuraminic acid) is not completely essential for GnRH binding. Furthermore, the solubilized GnRH receptor was less affected by wheat germ

Table II: Effect of Neuraminidase Treatment on ^{125}I-Buserelin Binding to Solubilized or Membrane-Associated GnRH Receptor[a]

neuraminidase (units/mL)	specific binding (%)	
	solubilized receptor	membranal receptor
0.01	100	94
0.05	100	80
0.1	90	76
0.25	82	50

[a] Solubilized or membrane-associated GnRH receptor was incubated (60 min at 22 °C) with different concentrations of neuraminidase in 1 mM CHAPS/10 mM Tris/0.1% BSA or 10 mM Tris/0.1% BSA, respectively, and subseuently binding assays to membranal or solubilized receptors were measured as described under Materials and Methods.

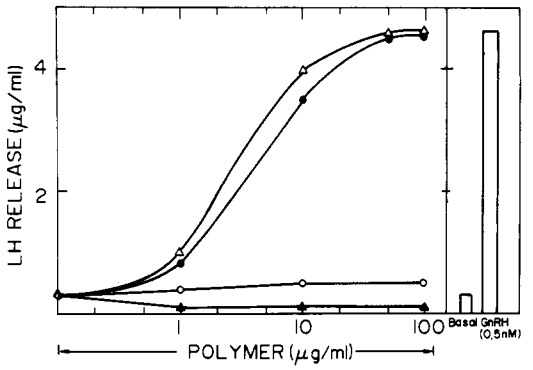

FIGURE 3: Dose-dependent effect of poly(amino acids) on LH release from pituitary cells. Pituitary cells, cultured for 48 h, were washed and incubated with various concentrations of polylysine (△, n = 1400; ●, n = 400), poly(glutamic acid) (○, n = 80; ▲, n = 120), or 0.5 nM GnRH. After 4 h at 37 °C, aliquots of the medium were taken for radioimmunoassay of LH as described under Materials and Methods.

agglutinin (a specific lectin for sialic acid and *N*-acetylglucosamine). Preincubation (60 min at 22 °C) of the membrane-associated GnRH receptor with WGA decreased the specific binding of ^{125}I-buserelin in a dose-dependent manner, reaching a plateau of 50% inhibition at 30 μg/mL [data not shown and Hazum (1982)]. In contrast, WGA affected ^{125}I-buserelin binding to the solubilized GnRH receptor only slightly (5%, 8%, 13%, and 15% inhibition at 14, 35, 75, and 140 μg/mL WGA, respectively). This finding further suggests that sialic acid residues are not absolutely required for GnRH binding. Nevertheless, it is possible that the differential effect of WGA on the binding of GnRH to the solubilized or membrane-associated receptors is due to its complex interaction with the plasma membrane (Cuatrecasas, 1973).

To examine whether a charge interaction is involved in the biological activity of GnRH, the effect of polylysine and poly(glutamic acid) on LH release was studied. As shown in Figure 3, polylysines stimulated LH release from cultured rat pituitary cells, whereas poly(glutamic acid) has no effect. The ED_{50} of the polylysines was 3–4 μg/mL, and the release of LH was with the same efficacy as that of GnRH (Figure 3). This suggests that the carboxylic groups of the receptor are a potential site for interaction with GnRH.

Discussion

The first step in GnRH action is its interaction with specific receptors at the surface of gonadotrope cells (Conn et al., 1981). Elucidation of the forces involved in the formation of the hormone–receptor complex requires acquisition of the structural components of the receptor. Chemical modifications of the membrane-associated GnRH receptor have indicated that two aromatic amino acid residues and two carboxylic groups of the receptor are probably essential for GnRH binding (Keinan & Hazum, 1985). According to those findings and on the basis of the spatial conformation of GnRH in solution (Shinitzky & Fridkin, 1976; Shinitzky et al., 1976), we have postulated a model for GnRH interaction with the membrane-associated receptor. However, due to the fact that the effects observed could result from alteration of the protein environment at the binding site, such studies are greatly aided by developing techniques for solubilization of the hormone binding protein from the membrane under conditions in which its hormone binding activity, characteristic affinity, and specificity remain intact.

Recently, we and other investigators have successfully solubilized GnRH receptors from rat and bovine pituitary using the zwitterionic detergent CHAPS (Perrin et al., 1983; Winiger et al., 1983; Capponi et al., 1984; Hazum et al., 1986, 1987). Studies with rat pituitary have shown that GnRH and GnRH analogues exhibited identical binding affinity for the solubilized and the membrane-bound receptors (Hazum et al., 1986), indicating that during solubilization the binding affinity and specificity of the receptor are not altered. The present findings further demonstrate that the binding properties of the solubilized GnRH receptor are retained. The time dependence and temperature dependence of hormone binding are similar to those observed in membrane-associated GnRH receptor (Clayton & Catt, 1981). Maximal specific binding is obtained at 4 °C, whereas at both 22 and 37 °C the maximum binding is reduced. The reduction in peptide binding at 37 °C can mainly be attributed to receptor instability, since preincubation at this temperature irreversibly abolished the specific binding. However, at 22 °C, there is no significant tracer or receptor degradation, suggesting a small reduction in binding affinity.

In addition to the resemblance in time and temperature dependence, the binding of ^{125}I-buserelin to both the solubilized and membrane-bound GnRH receptor is similarly inhibited by mono- and divalent cations. Since the potency of the divalent cations in inhibiting the binding correlates with their association constants for dicarboxylic compounds (Martell & Sillen, 1964), the participation of two carboxyl groups in the binding site may be inferred. The carboxyl groups are probably associated with either aspartic or glutamic acid but not with sialic acid residues, since the soluble receptor is only slightly affected by pretreatment with neuraminidase or WGA. Supportive evidence for this conclusion comes from the results obtained following purification of GnRH receptor on WGA–agarose (Hazum et al., 1986). It has been shown that two peaks of binding activity are present: one eluted in the void volume (no sialic acid) and the second eluted with the specific sugar. This suggests that the binding of the hormone to the solubilized receptor can occur in the absence of exposed sialic acid residues. Further confirmation for the participation of carboxyl groups in the binding site requires acquisition of structural data for the receptor.

It is well-known that carboxyl groups on protein may serve as negatively charged loci for recognition of positively charged cations. Therefore, we have tested the effect of polylysines and poly(glutamic acids) on the biological activity. In agreement with other investigators (Conn et al., 1984), we have shown that only cationic polymers can stimulate LH release with full efficacy, suggesting that simple charge interactions are the cause of LH release. According to these findings, we postulate that ionic interactions stabilized the formation of the hormone–receptor complex. Indeed, omission of the positively charged amino acid arginine in position 8 of GnRH leads to an analogue that is devoid of binding and biological activity (Perrin et al., 1980). In addition, substitution of arginine by a negatively charged amino acid, e.g., [Glu^8]GnRH, or neu-

tralization of the positive charge, e.g., [ω-NO_2-Arg^8]GnRH, diminished the biological activity (Hazum et al., 1977). However, replacement of arginine with basic amino acids such as homoarginine, ornithine, or lysine results in analogues with substantial biological activity (Yabe et al., 1974). Taken together, these results suggest that the driving force for the formation of the hormone–receptor complex is an ionic interaction between the amino acid arginine in position 8, which is positively charged, and the carboxyl groups in the binding site.

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REFERENCES

Capponi, A. M., Aubert, M. L., & Clayton, R. N. (1984) *Life Sci. 34*, 2139–2144.
Clayton, R. N., & Catt, K. J. (1981) *Endocr. Rev. 2*, 186–209.
Conn, P. M., Marian, J., McMillan, M., Stern, J., Rogers, D. C., Hamby, M., Penna, A., & Grant, E. (1981) *Endocr. Rev. 2*, 171–185.
Conn, P. M., Rogers, D. C., Seay, S. G., & Staley, D. (1984) *Endocrinology (Baltimore) 115*, 1913–1917.
Cuatrecasas, P. (1973) *Biochemistry 12*, 1312–1323.
Hazum, E. (1981a) *Endocrinology (Baltimore) 109*, 1281–1283.
Hazum, E. (1981b) *Mol. Cell. Endocrinol. 23*, 275–281.
Hazum, E. (1982) *Mol. Cell. Endocrinol. 26*, 217–222.
Hazum, E., Fridkin, M., Meidan, R., & Koch, Y. (1977) *FEBS Lett. 76*, 187–190.
Hazum, E., Schvartz, I., Waksmann, Y., & Keinan, D. (1986) *J. Biol. Chem. 261*, 13043–13048.
Hazum, E., Schvartz, I., & Popliker, M. (1987) *J. Biol. Chem. 262*, 531–534.
Heber, D., & Odell, W. D. (1978) *Biochem. Biophys. Res. Commun. 82*, 67–73.
Keinan, D., & Hazum, E. (1985) *Biochemistry 24*, 7728–7732.
Martell, A. E., & Sillen, L. G. (1964) in *Stability Constant*, Spec. Publ.—Chem. Soc. No. 70, Chemical Society, London.
Perrin, M. H., Rivier, J. E., & Vale, W. W. (1980) *Endocrinology (Baltimore) 106*, 1289–1296.
Perrin, M. H., Haas, Y., Rivier, J. E., & Vale, W. W. (1983) *Endocrinology (Baltimore) 112*, 1538–1540.
Sandow, J., & Konig, W. (1979) *J. Endocrinol. 81*, 175–182.
Schvartz, I., & Hazum, E. (1985) *Endocrinology (Baltimore) 116*, 2341–2346.
Shinitzky, M., & Fridkin, M. (1976) *Biochim. Biophys. Acta 434*, 137–143.
Shinitzky, M., Hazum, E., & Fridkin, M. (1976) *Biochim. Biophys. Acta 453*, 553–557.
Winiger, B. P., Birabeau, M. A., Lang, U., Capponi, A. M., Sizonenko, P. C., & Aubert, M. L. (1983) *Mol. Cell. Endocrinol. 31*, 77–91.
Yabe, Y., Kitamura, K., Miura, C., & Baba, Y. (1974) *Chem. Pharm. Bull. 22*, 2557–2564.

Enzyme Relaxation in the Reaction Catalyzed by Triosephosphate Isomerase: Detection and Kinetic Characterization of Two Unliganded Forms of the Enzyme†

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ABSTRACT: Triosephosphate isomerase has been shown to exist in two unliganded forms, one of which binds and isomerizes (*R*)-glyceraldehyde 3-phosphate and the other of which binds and isomerizes dihydroxyacetone 3-phosphate. The tracer perturbation method of Britton demonstrates the kinetic significance of the interconversion of these two enzyme forms at high substrate concentrations and yields a rate constant of about 10^6 s^{-1} for the interconversion. Although the molecular nature of the two forms of unliganded enzyme is not defined by these experiments, a shuffling of protons among active site residues, or a protein conformational change, or both, may be involved. This study, coupled with the known rate constants for the substrate-handling steps of triosephosphate isomerase catalysis, completes the kinetic characterization of the catalytic cycle for this enzyme.

Catalysis is a cyclic process. After facilitating the conversion of substrate to product, the catalyst must return to the form that accepts substrate so that the next cycle can begin. The regeneration of the substrate-accepting form of a catalyst may not be a trivial process, especially in enzymatic reactions where the transformation of substrate to product can involve a variety of molecular events (Walsh, 1979). At one extreme, relatively large-scale motions are believed to occur during catalysis by enzyme systems in which coenzymes are covalently bound to the protein and carry reaction intermediates from site to site. For example, in most carboxylases biotin is attached to the ε-NH_2 group of a lysine residue, and the cofactor appears to act as a mobile carboxyl group carrier between the catalytic sites of transcarboxylase (Northrop, 1969; Gerwin et al., 1969), of acetyl coenzyme A carboxylase (Guchhait et al., 1974a,b; Polakis et al., 1974), and of pyruvate carboxylase (Goodall et al., 1981). Analogously, lipoic acid residues are part of

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