0013-7227/89/1252-1025$02.00/0
Endocrinology

Vol. 125, No. 2
Printed in U.S.A.

Luteinizing Hormone-Releasing Hormone-Binding Sites in the Rat Thymus: Characteristics and Biological Function

B. MARCHETTI, V. GUARCELLO, M. C. MORALE, G. BARTOLONI, Z. FARINELLA, S. CORDARO, AND U. SCAPAGNINI

Department of Pharmacology and Second Department of Pathology (G.B., S.C.), Medical School, University of Catania, 95125 Catania, Italy

ABSTRACT. The present study was designed to explore the effects of LHRH and its agonists on immune system function. As a first step, to identify a putative site of action, the very potent and stable LHRH agonist (LHRH-A), [D-Ser(TBU^6)] des-Gly^{10}-LHRH ethylamide (buserelin), was used as an iodinated ligand to characterize LHRH receptors in a membrane preparation of rat thymus, a key organ of the immune system. The effects of LHRH and LHRH-A were then investigated on the proliferative capacity of rat thymocytes exposed *in vitro* to a mitogen and on ornithine decarboxylase specific activity. In addition, to determine whether LHRH-A treatment *in vivo* might directly influence thymic function, we treated hypophysectomized (hypox) rats with a moderately high dose of LHRH-A for a period of 2 weeks, and thymocyte mitogenic capacity, thymus weight, and the histological and functional appearance of the thymus were then assessed.

Specific binding of LHRH-A to rat thymic membrane preparations is a saturable process, depending on both time and temperature of incubation, but differs markedly from binding to the rat pituitary or ovarian LHRH receptor in its low binding affinity. Binding is optimal in the absence of chelating agents (EDTA) or divalent metal ions, and increases linearly with increasing protein concentration. Binding is specific for LHRH, LHRH-A, and antagonists. Both the C-terminal amide and N-terminal regions of the LHRH molecule were required for binding, and amino acid substitutions at position 6 markedly enhanced and at position 8 markedly reduced binding potencies in rat thymic tissue. A number of peptides, proteins, and other agents had no effect on the specific binding of LHRH-A to thymic membrane preparations. The binding affinity (K_a) of the membrane receptor of the rat thymus for the LHRH superagonist buserelin was 8.4×10^8 M^{-1}, while a higher binding affinity ($K_a = 2.8 \times 10^9$ M^{-1}) was calculated for the ovarian LHRH-binding site. Preincubation of rat thymocytes with LHRH-A for 20 h induced a significant dose-dependent increase in the proliferative response to the mitogen Concanavalin-A, monitored by [^{3}H]thymidine incorporation. Using native LHRH, it was also possible to elicit stimulatory effects on the same parameter, although much higher concentrations were required than with LHRH-A. Furthermore, simultaneous addition of a LHRH antagonist, abolished the LHRH effect on thymocytes. Ornithine decarboxylase specific activity under lectin stimulation was also significantly increased by LHRH-A in cultures of rat thymocytes. While in hypox rats a sharp inhibition of thymus weight and thymocyte proliferative capacity were observed, daily treatment of hypox rats with LHRH-A produced a 50% increase in thymus size accompanied by a significant stimulation of the cortical region of the thymus as well as a profound (~40 times) increase in thymus cell mitogenesis.

The present results indicate 1) the presence of a binding site which enables LHRH and its agonists to act upon a primary organ of the immune system; 2) that LHRH and its agonists directly influence *in vitro* a cell-mediated immune response; and 3) that LHRH-A treatment *in vivo* directly stimulates thymus weight and thymocyte mitogenesis without the participation of the pituitary gland, suggesting that LHRH or LHRH-like peptides as well as its potent agonists may exert important direct effects on immune system function. (*Endocrinology* **125:** 1025–1036, 1989)

THE HYPOTHALAMIC decapeptide LHRH directs and coordinates a complex series of biological events underlying the control of reproductive function. Accumulating evidence suggests that this small peptide may also act as a paracrine hormone and neurotransmitter in multiple extrapituitary sites via specific receptors localized in the brain (1, 2) and peripheral target organs, including the rat ovary (3–5) and testis (6, 7), the human placenta (8, 9), the human ovary (10–12), and human breast carcinoma (13, 14). Demonstration of LHRH or LHRH-like peptides in extrahypothalamic regions of the central nervous system and in peripheral tissues (15–18) clearly suggests the possibility of a physiological paracrine role involving locally produced LHRH factors.

On the other hand, accumulating data reveal that the central nervous system exerts a major regulatory control over the immune system, and that products of the immune system are capable of influencing the neuroendocrine axes via an action on neuronal cell groups or directly at the level of the pituitary and other peripheral

Received February 21, 1989.
Address all correspondence and requests for reprints to: Bianca Marchetti, Ph.D., Department of Pharmacology, Medical School, University of Catania, 6 A. Doria, 95125 Catania, Italy.

target cells (19–22). The mechanism for such reciprocal regulation appears to be that immune and neuroendocrine systems share a set of receptors and ligands (19, 20, 22).

Concerning the interconnections between the immune and reproductive systems, while the important modulatory role of gonadal hormones over the thymus gland has been known for at least a century (23), more recent studies have clearly shown the ability of the thymus to secrete hormones capable of regulating the reproductive axis (21, 24–26). Indeed, two thymic hormones, thymosin fraction 5 and one of its peptide constituents, thymosin β_4, are potent inducers of LHRH release from the hypothalamus, thus stimulating pituitary LH release (24, 25). Hence, thymic factors are able to influence reproductive physiology via an action at the level of hypothalamic LHRH machinery.

The present paper adresses the question of whether the hypophysiotropic hormone LHRH, one of the most important synchronizing factors involved in the regulation of reproductive processes (17, 27, 28), might communicate with immune cells, therefore influencing their activity.

Since binding to a specific receptor represents the first obligatory step in hormonal action, the presence of specific binding sites for LHRH in a primary organ of the immune system, the thymus, was investigated. To determine the biological function of the thymic LHRH receptor, the effects of LHRH and its potent agonistic (LHRH-A) and antagonistic analogs on the proliferative capacity of cultured thymic cells stimulated by a lectin mitogen were studied *in vitro*. In addition, to investigate the possibility of a direct effect of the peptide *in vivo*, hypophysectomized (hypox) rats were treated with LHRH-A, and thymus weight and histological appearance as well thymocytes proliferative capacity were assessed.

Materials and Methods

Animals

Adult (300- to 325-g) intact female and male and hypox Sprague-Dawley [Crl:CD(SD)Br] male rats were obtained from Charles River (Calco, Como, Italy) and housed two per cage under a regimen of 14 h of light and 10 h of darkness (lights on between 0600–2000 h). Purina rat chow and tap water (for intact animals) or 5% glucose in saline (for hypox rats) were available *ad libitum*. Experimental groups consisted of 18–20 animals.

Materials

[D-Ser(TBU^6)]Des-Gly^{10}-LHRH-*N*-ethylamide (buserelin) was a generous gift of Dr. J. Sandow (Hoechst AG, Frankfurt, West Germany). LHRH, the LHRH agonists [D-Trp^6]-, [D-Ala^6]-, and [D-Leu^6]des-Gly^{10}-LHRH-*N*-ethylamide; the LHRH antagonists D-*p*Glu^1,D-Phe^2,D-Trp^{3,6}-LHRH and LHRH-*N*-ethylamide; as well as TRH, arginine vasopressin, angiotensin-I, angiotensin-II, neurotensin, somatostatin, oxytocin, Met-enkephalin, hCG, LH, PRL, $CaCl_2$, $MgCl_2$, and EDTA were purchased from Sigma (St. Louis, MO), while LHRH-(3–10), [Gly^{10}]LHRH, and [Gln^8]LHRH, were obtained from Nova Biochemicals (Switzerland). Concanavalin-A (Con-A) was purchased from Pharmacia (Uppsala, Sweden), while RPMI, penicillin, and streptomycin were obtained from Gibco BRL (Scotland, United Kingdom), and Omnifluor was from New England Nuclear (Boston, MA).

Tissue preparation

For receptor studies, thymus glands were carefully removed, immediately placed on ice, cleaned, and weighed. All subsequent manipulations were performed at 4 C. Tissues were individually homogenized (1:30, wt/vol) in ice-cold 0.25 M sucrose, 50 mM Tris-HCl (pH 7.6) in a Dounce homogenizer and centrifuged twice at 800 × *g* for 5 min before centrifugation of the collected supernatants at 10,000 × *g* for 30 min. The 10,000 × *g* pellet was resupsended in assay buffer (30 mg tissue weight/ml) and used directly in the binding assay. Ovaries were processed similarly. The protein concentration was determined by the method of Lowry *et al.* (29), using BSA as a standard.

Receptor [^{125}I]LHRH-A assay

The very potent and stable LHRH-A, buserelin, was used for iodination and as a standard to measure thymic LHRH-binding sites. Buserelin was labeled with ^{125}I using the chloramine-T method, as previously described in full details (30, 31), at a specific activity of 600–1000 μCi/μg. The LHRH receptor assay was carried out according to the method of Marchetti *et al.* (30, 31) in a total volume of 500 μl for various times and temperatures (Fig. 1) and routinely for 90 min at 0–4 C. Assays were performed in triplicate in polypropylene test tubes, presoaked overnight in 1% albumin. To study ionic effects on LHRH-A binding (Figs. 2 and 3), the cations $CaCl_2$ and $MgCl_2$, and the cation chelator EDTA, pH 7.6, were included in the incubation mixture at concentrations of 0, 2, 5, 10, and 20 mM, while NaCl and KCl were used at concentrations of 10 and 100 mM, as indicated.

For displacement curves the assay mixture contained 200 μl buffer (50 mM Tris-HCl and 0.1% BSA, pH 7.6), 100 μl [^{125}I] buserelin (150,000 cpm; 0.5 nM), 100 μl unlabeled peptides at the indicated concentrations, and 100 μl membrane preparation of thymic or ovarian tissues. LHRH, LHRH fragments, LHRH-A, and antagonists as well as other peptides, including TRH, arginine vasopressin, angiotensin-I and -II, somatostatin, neurotensin, oxytocin, and Met-enkephalin were tested at concentrations of 10^{10}–10^4 M^{-1}, while hCG, ovine (o) LH, and oPRL were used in concentrations up to 10^6 M^{-1}. Routinely, a concentration of 10 μg/tube cold buserelin was used to measure nonspecific binding. Bound hormone was separated after washing with 3 ml ice-cold Tris-HCl buffer, pH 7.6, and by centrifugation at 15,000 × *g*. The supernatants were aspirated, and the radioactivity contained in the pellet was counted in a γ-counter at 70% efficiency.