Luteinizing hormone-releasing hormone (LH-RH) binding to purified rat pituitary nuclei

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1. INTRODUCTION

Fluorescently labelled LH-RH has been visualised as initially binding uniformly to the surface of pituitary gonadotrophs, then to aggregate, and finally become internalised [1,2]. Internalised LH-RH is thought to be sequestered and degraded by lysosomes [2] but may also become associated with other subcellular organelles and serve a regulatory function [3]. LH-RH stimulates LH release in a biphasic manner [4–6]; an initial rapid phase followed by a slower phase which is dependent on protein synthesis [4,6]. Since LH-RH stimulation of the anterior pituitary is known to increase the biosynthesis of gonadotrophic hormones [7] and the number of LH-RH receptors [8] it is possible that one of its sites of action is the nucleus. Internalisation and nuclear binding of peptide hormones has been demonstrated for insulin [9–11], EGF [12], NGF [13,14], LH [15] and thyrotropin releasing hormone [16]. Therefore, we have examined the binding of LH-RH and its analogues to purified anterior pituitary nuclei.

2. MATERIALS AND METHODS

2.1. *Preparation of nuclei*

Rats were decapitated, the anterior pituitary gland dissected out and diced in Tris–HCl 10 mM, EDTA 1.5 mM, BSA 0.1%, DTT 1 mM, pH 7.4 (TEBD) at 4°C. After several washings with this buffer the tissue was homogenized in a Dounce homogenizer (15 strokes), taken up in 20 ml buffer and centrifuged at $1000 \times g$ for 10 min to yield a crude nuclear pellet. The supernatant was centrifuged at $20000 \times g$ for 20 min to give a crude membrane preparation [17]. Purified nuclei were prepared by a method modified from [18,19]. The crude nuclear pellet was resuspended in a 2.3 M sucrose, 15 mM Tris–HCl, 25 mM KCl, 1.5 mM $MgCl_2$ (pH 7.5) and centrifuged at $18000 \times g$ for 10 min. The pellet was resuspended in the same buffer and centrifuged at $30000 \times g$ for 30 min. Phase contrast microscopy revealed a preparation of single unclumped nuclei and an apparent absence of contaminating subcellular material. This picture was confirmed by electron microscopy and the nuclei shown to be intact with both inner and outer nuclear membranes present. The total 5′-nucleotidase activity [20] in the nuclear preparation was 4% of that of the membrane fraction resulting from the same preparation. In some studies requiring processing of a large number of groups of pituitaries, purification of nuclei was achieved by centrifugation through 1.2 M sucrose (see table 3).

2.2. *Binding studies*

D-Trp^6–des-Gly^{10}–LH-RH-ethylamine (D-Trp^6-agonist) and D-Ala^6–*N*-Me-Leu^7–des-Gly^{10}–LH-RH-ethylamide (D-Ala^6-agonist) (gifts from J. Rivier) were radioiodinated with ^{125}I by the chloramine T method [21,22] and purified by Whatman CM32 carboxymethyl cellulose [21] and QAE Sephadex A-25 chromatography [23], respectively. Specific activity as determined by self-

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displacement from a specific antiserum [21] or from pituitary membranes varied from 990–1250 μCi/μg. The proportion of ^{125}I-LH-RH analogue bound by excess pituitary membranes was 33–40%. Preparations (containing 0.3–2.25 pituitary equivalents) were incubated with 70000 dpm ^{125}I-LH-RH agonist, and unlabelled LH-RH, LH-RH analogues or other peptides in 500 μl at 4°C for 90 min. Non-specific binding was determined by the addition of 10^{-6} M of the homologous LH-RH analogue. Incubation was terminated by the addition of 3 ml cold phosphate-buffered saline containing 1% BSA to the tubes and filtration through presoaked Whatman GF/C filters. Filters were washed 3 times with 3 ml PBS–BSA and counted in a gamma counter. All determinations were done in triplicate and the data presented as the mean.

3. RESULTS

Nuclear binding of LH-RH agonists comprised a single class of saturable, high affinity, specific sites similar to pituitary membrane receptors (fig. 1). Nuclear and membrane binding sites were similar in their interaction with the D-Ala^6-agonist, LH-RH and biologically inactive peptides (fig. 1, table 1). As a check on the validity of these estimations,

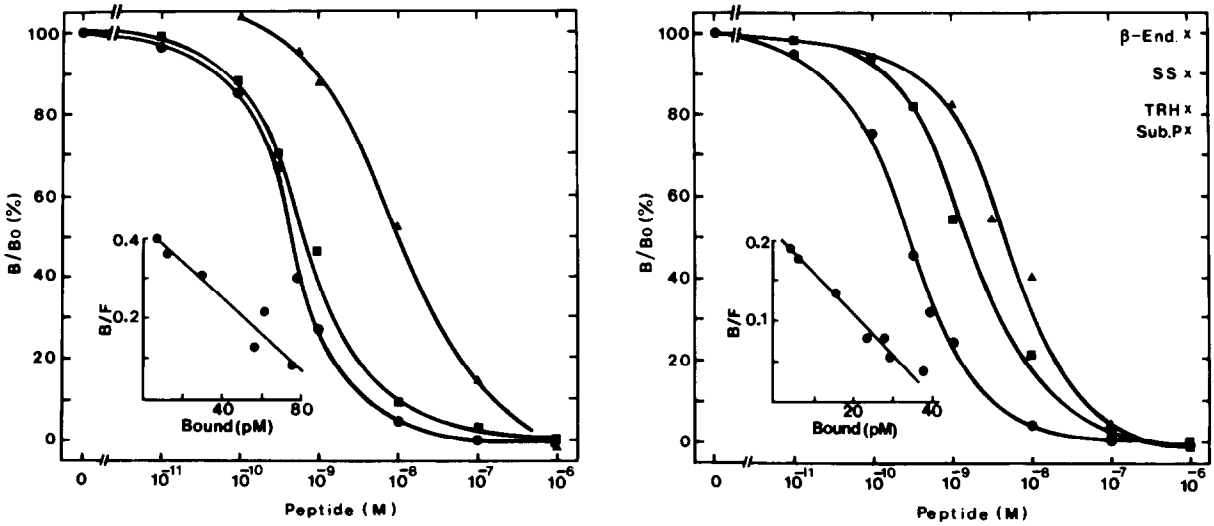

Fig. 1. An example of displacement curves of ^{125}I-D-Ala^6–*N*-Me-Leu^7-des-Gly^{10}–LH-RH-ethylamide by D-Ala^6–*N*-Me-Leu^7–des-Gly^{10}–LH-RH-ethylamide (●), an antagonist, D-*p*Glu^1–D-Phe^2–D-Trp^{3,6}–LH-RH (■) and LH-RH (▲) from a single study using male rat anterior pituitary membranes (left) and nuclei (right). Scatchard plots of the agonist (●) data are shown in the insets. Each point is the mean of 3 determinations using material derived from 2.25 pituitary equivalents.

Table 1

Apparent K_d-values of LH-RH and analogues

	Agonist D-Ala^6-*N*-Me-Leu^7–LH-RH-ethylamide	Antagonist D-*p*Glu^1–D-Phe^2–D-Trp^{3,6}–LH-RH	Native LH-RH
Membranes	0.30 ± 0.03 (5)	0.32 ± 0.06 (3)	9.7 ± 3.2 (3)
Nuclei	0.22 ± 0.03 (7)	0.66 ± 0.31 (4)	16.2 ± 14.2 (3)

K_d-values [nM; mean ± SEM (number of determinations)] were determined by Scatchard analysis or as in [24], and are the mean of values obtained from separate binding curves which employed both male and female rats. Mean binding of the D-Ala^6-agonist to membranes and nuclei was 29.7 ± 1.6 and 13.0 ± 1.7 fmol/pituitary equivalent, respectively

the K_d of ^{125}I-D-Ala^6-agonist was also estimated from the association (k_{on}) and dissociation (k_{off}) rate constants and found to be 3.4×10^{-10} M and 0.8×10^{-10} M for the membrane and nuclear preparations, respectively. Although the K_d of the LH-RH antagonist binding to nuclei was higher than that of the membranes (table 1), the difference was not significant and more extensive studies with LH-RH analogues are required to determine whether there are differences in the binding sites.

The possibility of contamination of the purified nuclei with plasma membranes containing LH-RH receptors was checked by quantitating 5′-nucleotidase activity. Specific binding of ^{125}I-LH-RH analogue relative to 5′-nucleotidase activity was 25-fold higher in the purified nuclear preparation. To further rule out the possibility that nuclear binding of LH-RH was due to contamination with other subcellular material, we investigated LH-RH binding by liver nuclei prepared along with pituitary membranes. Purified nuclei were prepared from rat liver, mixed with a pituitary membrane fraction ($1000 \times g$ supernatant) prepared from the same weight of anterior pituitary tissue, rehomogenized, and subjected to the usual purification procedures. Purified liver nuclei did not bind ^{125}I-LH-RH analogue but exhibited low but distinct binding after homogenization with the pituitary membrane fraction (table 2). When the nuclei were prepared in the presence of 0.001% Triton X-100 no binding was detectable in liver nuclei while pituitary nuclei retained 81% of the binding (table 2).

During the rat estrous cycle both nuclear and membrane binding was lowest at estrus and diestrus I and increased at diestrus II to reach highest levels during the morning of proestrus (table 3). There was no significant change in the affinity of nuclear or membrane binding sites for LH-RH during the estrous cycle. In these studies nuclear binding was determined using nuclei prepared by centrifugation through 1.2 M sucrose. Light microscopic examination of haematoxylin–eosin-stained preparations indicated that the nuclei were uncontaminated. However, since we have not

Table 2

Specific binding (cpm) of ^{125}I-D-Trp^6–des-Gly^{10}–LH-RH-ethylamide to pituitary and liver nuclei

Triton	Pituitary nuclei	Liver nuclei		Supernatant of liver; nuclei prepared with pituitary membranes
		− pituitary membranes	+ pituitary membranes	
−	4157 + 381	99 + 155	680 + 174	2724 + 401
+	3379 + 434	76 + 182	150 + 56	2257 + 312

Nuclei were prepared from Long Evans male rat liver and pituitary as described in the text

Table 3

Nuclear and membrane binding of LH-RH agonist during the rat estrous cycle

	K_d (nM ± SEM)		Q (fmol/pituitary)	
	Membranes	Nuclei	Membranes	Nuclei
Diestrus I	0.44 ± 0.10	0.29 ± 0.07	38.3 ± 2.8	19.0 ± 4.0
Diestrus II	0.45 ± 0.14	0.23 ± 0.04	61.5 ± 5.1	32.7 ± 4.3
Proestrus	0.35 ± 0.03	0.25 ± 0.08	81.7 ± 7.7	49.1 ± 7.6
Estrus	0.30 ± 0.06	0.26 ± 0.03	35.2 ± 5.8	16.8 ± 2.6

Adult female Wistar rats were housed under a 14:10 h light:dark cycle. The stage of the estrous cycle was determined from vaginal smears. Between 24 and 45 females for each stage were decapitated at 10 a.m. Nuclei were prepared from the $1000 \times g$ pellet by two successive resuspensions in fresh TEBD buffer and centrifugation at $1000 \times g$ followed by centrifugation through 1.2 M sucrose TEBD at $9000 \times g$ for 45 min. Data were calculated from Scatchard analysis of binding of the D-Ala^6-agonist

fully characterised nuclei prepared in this way by enzyme marker and electron microscopic studies it is possible that a small proportion of the nuclear binding might be due to contaminating subcellular particulate material.

4. DISCUSSION

This study has demonstrated the presence of high affinity, specific nuclear binding sites for LH-RH in the rat anterior pituitary which have properties similar to those of LH-RH membrane receptors. Since this binding could have been due to an association of LH-RH receptors in plasma membranes or other subcellular material with the nuclei during preparation we have undertaken studies to investigate this. A number of points argue against the possibility of significant contamination of the nuclei with non-nuclear LH-RH binding material. The nuclei were prepared by methodology previously shown to produce a purified preparation [9–11,18,19] and bound LH-RH with a substantial capacity. The purity of these nuclei was established by electron microscopic examinations. The specific binding of ^{125}I-LH-RH analogue to nuclei relative to 5′-nucleotidase activity indicates that the binding is not due to contamination with plasma membrane receptors. In other studies we demonstrated that purified liver nuclei bind LH-RH poorly when prepared with pituitary membranes (16% relative to pituitary nuclei) and that the majority of the membrane binding remained unassociated with the liver nuclei. When liver nuclei were prepared in the presence of pituitary membranes with 0.001% Triton X-100 this binding was abolished while pituitary nuclear binding was still substantial. The site of LH-RH binding to the nucleus may be the chromatin or nuclear membranes. However, cytoskeletal filaments have not been excluded as possible LH-RH binding sites in our experiments since they are an integral part of nuclei purified by both detergent and mechanical lysis, and are not easily revealed by electron microscopy [25]. Using conditions which remove all membranous material (2% Triton X-100) both nuclear and membrane binding of LH-RH were completely lost (not shown).

The degree of LH-RH binding by purified nuclei represented 33.1 ± 3.6% of the total binding of the pituitary which is somewhat higher than the relative binding of insulin (10%) to isolated nuclei [9]. However, the binding of TRH to purified pituitary nuclei (15–20% of total cellular binding) [16] is similar to that of LH-RH.

During the rat estrous cycle binding of LH-RH to pituitary membranes increased on the morning of proestrus [26] and the nuclear preparations exhibited a similar pattern. The findings do not exclude the possibility, however, that nuclear LH-RH binding might be different from that of membrane receptors during the afternoon of proestrus when membrane binding declines just prior to the preovulatory gonadotropin surge [27].

The role of LH-RH internalisation is unknown but the decrease in receptor number accompanying stimulation with high concentrations of LH-RH may be explained by internalisation, separately, or in conjunction with, sequestration and degradation by lysosomes [2]. Internalisation of LH-RH could also be related to binding to, and regulation of, subcellular organelles such as the nucleus, cytoskeletal filaments and secretory granules. LH-RH binding to nuclei might be responsible for the late events in LH-RH action, mediated via increased protein synthesis as suggested for insulin [9]. Since the late phase of LH-RH-stimulated gonadotrophin release is impaired by inhibitors of protein synthesis [4,6] it is possible that LH-RH association with nuclei might serve a role in stimulating de novo synthesis of these hormones. Another late event in LH-RH action which may be dependent on LH-RH binding to nuclei is the stimulation of an increase in LH-RH membrane receptors on the pituitary gonadotroph [8].

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