INTERACTION OF $^{125}$I-LH-RH AND OTHER Oligopeptides With Plasma Membranes Of Rat Anterior Pituitaries

By

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ABSTRACT

The specific binding of $^{125}$I-LH-RH to isolated plasma membranes of rat pituitaries was investigated. The binding process was found to be highly specific, temperature-dependent and saturable. The dissociation constant as calculated by three different methods was approximately $1.3 \cdot 10^{-8}$ M, indicating a single type of binding sites. Maximal binding capacity was $1 \cdot 10^{-12}$ moles/mg protein (= 2 mg LH-RH/pituitary gland), and the number of binding sites was calculated to be $6 \cdot 10^{11}$ per mg membrane protein (= $1 \cdot 10^{10}$ binding sites/pituitary gland). When diluted with ice-cold buffer the dissociation of specifically bound LH-RH occurred very rapidly (half-life 3.17 min) with a rate constant of 0.219 min$^{-1}$. The dissociation process followed first-order kinetics. Specificity of binding was demonstrated by dose-dependent competition of unlabelled LH-RH, the highly potent analogue D-glutamine-(cyclo-hexyl)$^6$-LH-RH-nonapeptide-ethylamide and the fragment of an analogue (6-D-Ser(TBu))-LH-RH-(3-9)-heptapeptide-ethylamide with the binding $^{125}$I-LH-RH, while angiotensin I, II, oxytocin and bacitracin did not compete. The affinities of LH-RH and the analogue to the binding sites of the pituitary plasma membranes were not consistent with the respective biological activities.

The initiation of the release mechanism for both LH and FSH from the pituitary gonadotrophs is in all probability a function of the binding of LH-RH to specific receptors.
Although binding sites for LH-RH have been found on dispersed pituitary cells (Grant et al. 1973; Hopkins & Gregory 1977; Zolman & Valenta 1978), in pituitary homogenate (Spona 1973; Pedroza et al. 1977), in pituitary plasma membrane preparations (Spona 1973, 1974; Marshall et al. 1976; Théoleyre et al. 1976), and on secretory granules (Sternberger & Petrali 1975; Sternberger & Hoffman 1978; Sternberger et al. 1978), the exact location of the LH-RH receptors mediating the events leading to the exocytosis of the gonadotrophin containing granules is still unknown.

Moreover, there are considerable discrepancies of opinion as to the types of binding sites identifiable in pituitary tissue. Contrary to Théoleyre et al. (1976) who ascertained by means of Scatchard plot that there is only a single type of binding, Spona (1973) and Marshall et al. (1976) found two different binding sites for LH-RH, one of high affinity and low capacity and another one of low affinity and high capacity. Zolman & Valenta (1978) actually obtained “multisigmoid” binding curves when increasing concentrations of iodine-labelled LH-RH were incubated with bovine pituitary plasma membranes.

In view of these contradictory results we investigated the binding properties of [125I]-LH-RH to isolated plasma membranes of rat pituitaries.

MATERIAL AND METHODS

Substances

Freshly labelled [125I]LH-RH was kindly supplied by Dr. Sandow (Hoechst AG, Frankfurt, FRG). Its biological activity, i.e. its capacity to release LH in ovariectomized, oestrogen-progesterone treated rats was shown to be 90% of that of non-labelled hormone (Niemann & Sandow 1973). For the binding experiments [125I]LH-RH was used within two weeks after iodination.

The specific activity of [125I]LH-RH was 210 to 230 mCi/mg. LH-RH, (6-D-Ser(TBu))-LH-RH(3-9)-heptapeptide-ethylamide, and (6-D-Gln(cyclohexyl)-LH-RH-(1-9)-nonapeptide-ethylamide were also gifts of Hoechst AG Frankfurt, FRG.

Bovine serum albumin (BSA) was obtained from Behringwerke, Marburg, FRG.

Preparation of plasma membranes

Plasma membranes were isolated from anterior pituitaries of adult female SIV rats (Kisslegg/Allgäu, FRG) weighing 180-250 g by means of differential centrifugation on a stepwise sucrose gradient. Immediately after decapitation the anterior pituitaries were removed and homogenated (1:10 w/w) in ice-cold Tris-HCl-buffer (0.05 M, pH 7.4) containing 0.25 M sucrose and 1 mM dithiothreitol in a Potter-Elvehjem-homogenizer (7 strokes), diluted with the same buffer (1:1) and centrifuged at 700 × g for 10 min. The supernatant was spun at 2000 × g for 30 min, the pellet suspended in the same buffer containing additionally 1 mM MgCl₂ and centrifuged at 40 000 × g for 40 min. Pellets consisting of material of 50 pituitaries each were re-suspended in 2 ml of Tris-HCl-buffer (0.05 M, pH 7.4) containing sucrose (density 1.14) and layered on a discontinuous sucrose gradient with 2 ml steps of 1.18, 1.20, 1.22, and 1.24 density.

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Centrifugation was carried out for 2 h at 115,000× g. The fractions obtained from each sucrose density interface were pooled, diluted with Tris-HCl-buffer (0.05 M, pH 7.4; 1 mM dithiothreitol, 1 mM MgCl₂) to 10 ml and centrifuged for 30 min at 150,000× g. The pellets were stored in liquid nitrogen. Prior to use in the binding assays, the pellet was suspended in Tris-HCl-buffer (0.05 M, pH 7.4; 1% BSA) to a concentration of 0.6 to 0.9 mg protein/ml.

Protein content was determined by means of a modification of the method of Lowry et al. (1951).

The binding studies were performed with membranes of the 1.18/1.20 density fraction which had been shown by means of electron microscopy to contain mainly plasma membranes. This fraction was found to have the highest specific binding capacity for [¹²⁵I]LH-RH, and to have the highest NaF-stimulatable adenyl cyclase activity (300 % increase by 10 mM NaF).

**Binding experiments**

All determinations were performed in quadruplicate at 0°C and at an incubation time of 30 min, unless stated otherwise. The incubations (final volume 70 µl) were carried out in conical 2 ml polystyrol tubes. The experiments were started by the addition of 30 to 50 µg of plasma membrane protein in 50 µl to approximately 400 pg [¹²⁵I]LH-RH (= 100,000 cpm) unless stated otherwise. Specific binding was evaluated by the presence of 11.8 µg of non-labelled LH-RH, except the experiment on the dependency of specific binding upon the concentration of labelled hormone when a 30,000-fold excess of LH-RH was used. The incubation was terminated by the addition of 1 ml of ice-cold Tris-HCl-buffer (0.05 M, pH 7.4; 1% BSA) and bound [¹²⁵I]LH-RH was immediately separated from free hormone by filtration through cellulose acetate filters (Millipore EHWP 02500). The filters had been pre-treated with 1% BSA, and showed a reproducible 0.5% adsorption of labelled hormone. These values were eliminated when non-specific binding was subtracted from total binding to give specific binding values. The filter was washed four times with 1 ml of ice-cold buffer. The procedures of filtration and washing were performed rapidly (approximately within 20 seconds) as it was found that bound [¹²⁵I]LH-RH dissociated rapidly even at 0°C from its “receptors” when the incubation mixture was diluted with 2 ml of cold buffer. After washing the filters were counted in a Packard Gamma Scintillation spectrometer.

**Results**

The interaction of hormones and binding sites located on plasma membranes involves the so-called specific and non-specific binding. Background and non-specific binding were usually determined by the addition of excessive non-labelled hormone to the labelled hormone prior to the addition of membrane protein. Specific binding is determined by subtracting non-displaceable binding from total binding. In order to determine the suitable excess of LH-RH, increasing concentrations of non-labelled LH-RH were added to 400 pg [¹²⁵I]LH-RH during the incubation with 30 µg membrane protein. Fig. 1a shows that noticeable displacement of labelled LH-RH occurred with 1.43 (10⁻⁶ M/l) LH-RH, and about 71 % of the total binding was displaced when 143 (10⁻⁶ M/l)
were added. At this point the displacement curve turns to an asymptotic form. Therefore, this excess of non-labelled hormone was utilized during further incubations for the evaluation of specific binding. The binding specificity was also demonstrated by the finding that the oligopeptides angiotensin I, oxytocin and bacitracin did not appreciably compete with ([125I]LH-RH for binding to pituitary plasma membranes. Similarly, the addition of 143 (10^{-6} M/l) TRH or of 10 µg of LH and FSH, and 5 µg of ACTH to the incubation mixture did not displace the labelled LH-RH from the binding sites.

Fig. 1.

a) Effect of increasing concentrations of LH-RH, angiotensin I, oxytocin, and bacitracin upon the binding of [125I]LH-RH to pituitary plasma membranes at 0°C. b) Specific binding of [125I]LH-RH to pituitary plasma membranes at 0°C and 37°C as a function of incubation time (mean ± SD).
The binding of LH-RH to pituitary plasma membranes is a time-dependent process. When the incubations were carried out at approximately 0°C, specific binding reached a plateau within 30 min (Fig. 1b). At a temperature 37°C, maximal binding was observed after 5 to 10 min, and a subsequent decline thereafter. The binding capacity was much higher at the low temperature as compared to 37°C.

The dependency of the interaction of LH-RH with pituitary plasma membranes upon the temperature is also demonstrated in Fig. 2a. Specific binding
was maximal at 0°C, and decreased remarkably when the temperature was elevated. At temperatures between 30°C and 35°C only non-specific binding remained after 30 min of incubation.

The specific binding of [125I]LH-RH to plasma membranes is nearly proportional to the amount of protein within the range used in the present study.

**Fig. 3.**

a) Specific binding of [125I]LH-RH to pituitary plasma membranes at 0°C as a function of the concentration of labelled hormone. b) Dissociation of specifically bound [125I]LH-RH after dilution at 0°C in dependency of time. After 30 min of incubation at 0°C the incubation mixture was diluted with 2 ml of ice-cold buffer, and the incubation was continued for various time intervals. Specific binding (●—●) was evaluated by subtracting non-specific binding (○--○) from total binding (●—●) (mean ± sn).
a) Scatchard plot of the specific binding of $[^{125}\text{I}]\text{LH-RH}$ to pituitary plasma membranes. The data are calculated from the experiment depicted in 3a. b) Evaluation of the dissociation constant $K_D$ and the maximal binding capacity $B_{\text{max}}$ of the specific binding of $[^{125}\text{I}]\text{LH-RH}$ to pituitary plasma membranes by means of the method of Eisenthal & Cornish-Bowden (1974). The values were obtained from Fig. 3a. ($B$ = specifically bound $[^{125}\text{I}]\text{LH-RH}$, $F$ = free $[^{125}\text{I}]\text{LH-RH}$).
The experiments were carried out at protein amounts ranging between 30 and 50 μg per incubation.

The specific binding of LH-RH to pituitary plasma membranes, as a function of labelled hormone concentration, is a saturable process (Fig. 3a). Saturation is nearly complete at a concentration of $25 \cdot 10^{-9}$ M $[^{125}\text{I}]$LH-RH. The dissociation constant was $1.295 \pm 0.106 \cdot 10^{-8}$ M as determined by the Scatchard plot (Scatchard 1949) (Fig. 4a), $1.37 \pm 0.20 \cdot 10^{-8}$ M by the method of Eisenthal & Cornish-Bowden (1974) (Fig. 4b), and $1.33 \pm 0.31 \cdot 10^{-8}$ M when the Lineweaver-Burk plot (Lineweaver & Burk 1934) (Fig. 5a) was applied. The maximal binding capacity of the plasma membranes for LH-RH was found to be $1.04 \pm 0.05 \cdot 10^{-12}$ moles/mg protein (Scatchard 1949), $1.0 \pm 0.17 \cdot 10^{-12}$ moles/mg protein (Eisenthal & Cornish-Bowden 1974), and $1.0 \pm 0.23 \cdot 10^{-12}$ moles/mg protein (Lineweaver & Burk 1934). The graphical methods used indicate a single type of binding site and no cooperativity. The number of binding sites was calculated from the value of maximal binding of LH-RH to be $6 \cdot 10^{11}$ per mg protein. This indicates a binding capacity of $2 \cdot 10^{-14}$ moles and a number of $1 \cdot 10^{10}$ binding sites for one pituitary.

The amount of $[^{125}\text{I}]$LH-RH specifically bound to pituitary membranes represented approximately 3 % of the free labelled hormone, i.e., when 100 000

![Graphs](image-url)

**Fig. 5.**

a) Lineweaver-Burk plot of the specific binding of $[^{125}\text{I}]$LH-RH to pituitary plasma membranes. The data are calculated from the values in Fig. 3a. b) Determination of the half-life time of $[^{125}\text{I}]$LH-RH specifically bound to pituitary plasma membranes. The data are calculated from the experiment described in Fig. 3b.
a) Substitution of the data depicted in Fig. 1b. into a second-order equation for the calculation of the association rate constant \( k_+ \) of specific binding of \(^{[125I]}\text{LH-RH} \) to pituitary plasma membranes at 0°C. (\( R_0 \) = initial concentration of free receptors; LH-RH\(_0 \) = initial concentrations of free \(^{[125I]}\text{LH-RH} \); B = concentrations of bound \(^{[125I]}\text{LH-RH} \). b) Effect of increasing concentrations of LH-RH, (6-D-Gln(cyclohexyl))\text-LH-RH-(1-9)-nonapeptide-ethylamide, (6-D-Ser(TBu))-LH-RH-(3-9)-heptapeptide-ethylamide, and angiotensin II upon the binding of \(^{[125I]}\text{LHRH} \) to pituitary plasma membranes at 0°C.
CPM of $[^{125}I]$LH-RH was added to the membrane mixture, the total binding was 4500 CPM, the non-displaceable non-specific binding 1350 CPM, and the specific binding about 3150 CPM.

The rate of dissociation ($k_-$) of the hormone-receptor complex at 0°C was measured by means of 30-fold dilution of the incubation mixture with ice-cold buffer at increasing time intervals (Fig. 3b). The dissociation occurred very rapidly within the first 5 min and was shown to be a first-order process with a half-life of the complex of 3.17 min (Fig. 5b). The rate constant of dissociation ($k_-$) was calculated to be 0.219 ± 0.012 min⁻¹.

In order to calculate the association-rate constant ($k_+$) the values along the curve at 0°C in Fig. 1b were substituted into a second-order equation as depicted in Fig. 6. The resulting curve did not reveal, however, that the association process followed second-order kinetics. This is probably due to the rapid dissociation.

In an additional experiment a biologically potent analogue of LH-RH and the (3-9)-fragment of another LH-RH-analogue were tested for their ability to interfere with the specific binding of labelled LH-RH to plasma membranes as compared to LH-RH and angiotensin II (Fig. 6b). While angiotensin II did not compete with $[^{125}I]$LH-RH for the binding sites, the highly potent analogue D-glutamine-(cyclohexyl)⁶-LH-RH-nonapeptide-ethylamide which had been shown in rats to be about 85 times as effective in the biological activity as LH-RH (Sandow et al. 1978), showed nearly the same binding capacity as LH-RH. Contrary to this, the (6-D-Ser(TBu))-LH-RH-(3-9)-heptapeptide-ethylamide which showed only 10% of the effect of LH-RH on gonadotrophin release in rats, had less than one third of the affinity to pituitary plasma membranes than that of LH-RH.

**DISCUSSION**

The results of the present study on the interaction of $[^{125}I]$LH-RH with isolated pituitary plasma membranes revealed that there is a single type of binding site. The binding process was shown to be highly specific, temperature-dependent and saturable, and the bound hormone was found to dissociate rapidly.

The specificity of the binding sites for LH-RH was demonstrated by the displacement of bound $[^{125}I]$LH-RH by LH-RH and some related hormones while some other oligopeptides, angiotensin I and II, oxytocin, TRH, and bacitracin, as well as the gonadotrophins did not compete with labelled LH-RH even at higher concentrations. The displacement of bound $[^{125}I]$LH-RH by the non-labelled releasing hormone was dependent on the concentration of LH-RH, and non-specific binding was estimated to be ascertained by the addition of a 30 000-fold excess of LH-RH.
The dissociation constant was calculated by means of three different methods to be in the order of $1.3 \cdot 10^{-8}$ M. Similar results had been reported by Marshall et al. (1976) and Spona (1973) who found $K_d$ to be $0.5 \cdot 10^{-8}$ M and $2 \cdot 10^{-8}$ M, and by Théoleyre et al. (1976) whose binding studies at 37°C revealed a constant of about $1 \cdot 10^{-8}$ M. While Théoleyre et al. (1976) in accordance with our results found out only one type of binding, Marshall et al. (1976) and Spona (1973) found an additional binding site with much lower affinity, $1.2 \cdot 10^{-6}$ M and $2 \cdot 10^{-7}$ M, respectively.

The contradictory results are, in all probability, due to different experimental conditions. As it was mentioned above, we utilized a 30 000-fold excess of non-labelled hormone to distinguish specific from non-displaceable binding. Contrary to this, Marshall et al. (1976) used a 2 million-fold excess, Théoleyre et al. (1976) a 1000-fold, and Zolman & Valenta (1978) who reported "multi-sigmoid" binding curves, a 100-fold excess.

There were also differences in the technique of the separation of bound from free hormone. It was carried out by Marshall et al. (1976) and Théoleyre et al. (1976) by means of centrifugation at 11 000 x g and 24 000 x g, while Spona (1973), Zolman & Valenta (1978), Grant et al. (1973), and our group preferred the filtration method. When filtered through Millipore filters, there is some adsorption of labelled hormone by the filters, and this is a potential source of error (Théoleyre et al. 1976). This problem is eliminated, however, by subtracting non-displaceable binding.

As is was demonstrated in the present study that the dissociation of the bound hormone occurs very rapidly after dilution at 0°C, a change of experimental conditions as described by Théoleyre et al. (1976), i.e. incubation at 37°C followed by dilution with ice-cold buffer and centrifugation at 0°C appears to be somewhat problematic.

During the initial 5 min of incubation after dilution the dissociation strictly follows first-order kinetics with a dissociation rate constant of $0.219$ min$^{-1} = 0.37 \cdot 10^{-2}$ seconds$^{-1}$ at 0°C. A similar value of $0.7 \cdot 10^{-2}$ seconds$^{-1}$ was observed by Théoleyre et al. (1976) at 37°C. The half-life time of the complex of 3.17 min was in good agreement with the findings of Spona (1973) who recorded a 42% dissociation after dilution for 2 min.

In contrast to our results, Spona (1973) could not demonstrate first-order kinetics, possibly because the dissociation of total binding was measured while we determined that of specific binding.

The association process of LH-RH to the binding sites did not appear to follow second-order kinetics. This may be due to the rapid dissociation which impairs the graphical estimation by means of a straight line when $\log R_o(LH$-RHe-B)/LH-RH0(R0-B) is plotted versus time. The method is based on the assumption that the back reaction is sufficiently small to be justifiably neglected (Snell et al. 1965). This was, however, clearly not the case.
The maximal binding capacity of $1 \cdot 10^{-12}$ moles/mg protein at $0^\circ C$ corresponds to $2 \cdot 10^{-14}$ moles/pituitary or to approximately 2 ng of LH-RH. Maximal binding capacity of isolated plasma membranes at $37^\circ C$ was calculated by Théoleyre et al. (1976) to be approximately $1 \cdot 10^{-13}$ moles/mg protein.

When the binding experiments were performed at $0^\circ C$, a plateau of binding was reached after about 30 min of incubation. In contrast, there was a marked decrease in the specific binding between 10 and 15 min of incubation at a temperature of $37^\circ C$. A similar phenomenon was observed by Théoleyre et al. (1976), and was interpreted to be the result of a partial denaturation of the receptors at this temperature. Preliminary results indicate, however, that this phenomenon is probably due to enzymatic degradation of the labelled hormone at $37^\circ C$ which takes place during incubation with isolated pituitary plasma membranes (Clayton et al. 1978; Sandow et al. 1979).

It has previously been shown by Kuhl et al. (1977, 1979) that the rat pituitary contains an enzyme system which is capable of degrading LH-RH very rapidly. Although this enzyme occurs mainly in the supernatant of pituitary homogenate, its intracellular origin is not known. Plasma membranes of various tissues have been shown to be capable of inactivating several oligopeptide hormones (Rodbell et al. 1971; Freychet et al. 1972; Marx et al. 1972).

When LH-RH was compared to the highly active analog D-glutamine (cyclohexyl)-6-LH-RH-nonapeptide-ethylamide with respect to its capability to compete with $[1^{25}]$I-LH-RH for membrane binding sites, no increased binding of the analogue could be observed although it was shown to be 80 times as effective in releasing gonadotrophins as compared to LH-RH. Similarly, Nair et al. (1978) reported no difference in the binding of highly potent LH-RH analogues and LH-RH to pituitary plasma membranes. It was also shown by Spona (1974) that an LH-RH analogue antagonizing LH-RH-mediated gonadotrophin release in vivo (Coy et al. 1973), possessed the same affinity to plasma membrane receptors as LH-RH. Using pituitary plasma membranes, Pedroza et al. (1977) demonstrated that at $4^\circ C$ the displacement of labelled LH-RH by the superactive analogue (D-Trp)-6-LH-RH is less than that produced by LH-RH itself. Contrary to this, incubations with pituitary homogenate or slices revealed a much greater potency of the analogue to bind to the receptors as compared to LH-RH, provided that degradation of labelled LH-RH was inhibited by the addition of bacitracin.

Using dispersed rat anterior pituitary cells, Grant et al. (1973) found out that LH-RH and various analogues show binding properties similar to the known biological activities.

It was proposed that LH-RH acts like large protein and polypeptide hormones on gonadotrophin release by a mechanism involving binding of the releasing hormone to receptors on the cell surface. The discrepancy in the
binding of LH-RH as compared to some analogues and in the biological effects of these peptides, however, gives some doubt upon the significance of the binding to isolated plasma membranes. Alterations of the structure of the binding units on the membranes may occur during the isolation procedure, as intact pituitary cells and slices show this parallelism of binding and biological effectiveness.

It remains to be shown, however, whether the binding sites on isolated pituitary plasma membranes are identical with the receptors which are involved in the pathway of LH-RH-mediated gonadotrophin release.

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