PITUITARY BINDING SITES FOR [^{3}H]-LABELLED LUTEINIZING HORMONE RELEASING FACTOR (LRF).

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SUMMARY: Normal rat anterior pituitary cells in culture possess two binding sites for [^{3}H]LRF. One is a high affinity (2×10^{-9}M), low capacity site which corresponds to the half-maximal biological potency of LRF. The second site has low affinity (2×10^{-8}M) and an enormously higher capacity to bind LRF. This second site shows only partial specificity in that inactive LRF-analogues, although not TRF, compete for binding of [^{3}H]LRF. The high affinity site is competible by LRF, LRF-agonists and LRF-antagonists even in the presence of an excess of an inactive LRF-analogue which saturates the low affinity binding site.

The interaction of LRF with its pituitary receptor has been discussed with respect to the biological potencies and intrinsic activities of various synthetic LRF analogues (1, 2). The effect of an LRF peptide on pituitary gonadotropic cells is an integrated process resulting in the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (3).

It has been established that thyrotropin releasing factor (TRF) acts at the level of the pituitary plasma membrane to initiate the release mechanism of stored thyroid stimulating hormone (TSH) (4-6). Although it has been postulated (7) and some evidence indicates that the thyrotropic and gonadotropic cells may be similar enough to be expected to possess phylogenetically related receptors, no direct experiments examining the characteristics of binding of labelled LRF to the receptors of gonadotropic cells have been reported. *In vitro* assay of the potency of LRF gives an apparent affinity constant of ca. 0.5×10^{-9}M LRF (8).

Although only a few analogues have been made which possess potencies close to equal that of LRF, several have been synthesized which competitively

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antagonize the action of LRF (2) and have either lower or no detectible intrinsic activity. It has been deduced from the biological activity data that most of the LRF agonistic analogues have lower affinity constants but full intrinsic activity for the LRF receptor, while at saturating concentrations the LRF antagonists are unable, or at least only partially able, to activate a response. At the level of the receptor this would suggest that all the analogues examined act on the same physiological LRF receptor.

This study was carried out to substantiate our deductions about the biological activity data and to characterize the specificity of [^{3}H]LRF binding to the LRF receptor at the membrane level.

MATERIALS AND METHODS

Peptide synthesis

The tritiated luteinizing hormone releasing factor [^{3}H-Pro^9]LRF was synthesized by solid-phase methodology on a benzhydrylamine resin. The general synthetic approach and purification methods used here have been described by Rivier et al. (9) and Monahan and Rivier (10) for LRF and analogues. After purification a homogeneous decapeptide (final radioactive specific activity 35.6 C/mmole) was obtained; it possessed the correct amino acid ratio for LRF. Peptides were stored frozen in dilute acetic acid.

Biological potencies of LRF, labelled LRF and LRF analogues were determined in vivo and in vitro. [^{3}H-Pro^9]LRF had maximal biological activity based on the quantitation of radioactivity and amino acid content.

Anterior pituitary cells

Pituitary cells were dispersed and prepared as reported by Vale et al. (8). After several days in culture the cells were removed from the dish by addition of 10^{-3}M EDTA in Hepes buffer with glucose (5 minutes) followed by gentle scraping of the dish with a rubber policeman.

[^{3}H]LRF binding

The experiments were carried out essentially as those reported for

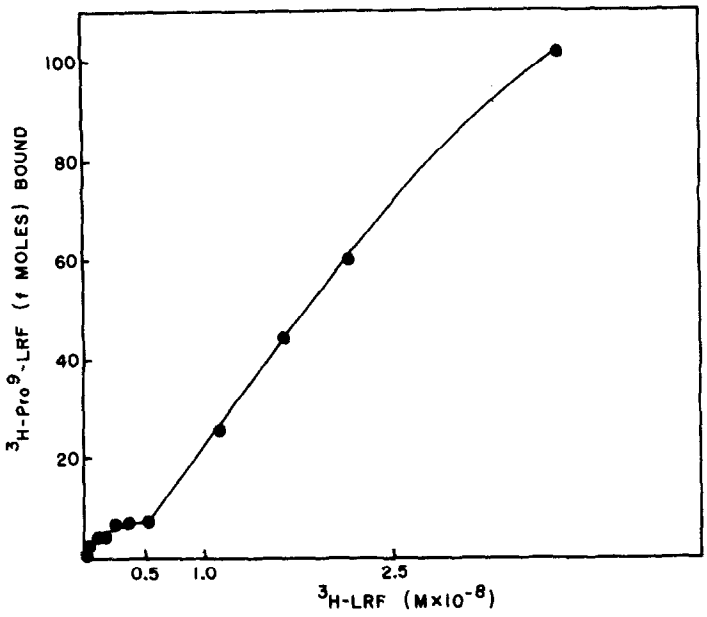

Figure 1. Binding curve of [^{3}H-Pro^9]LRF to dispersed cultured normal rat pituitary cells. Non-competible binding was controlled by addition of excess (1 mM) LRF to the incubation mixture.

[^{3}H]TRF binding (4). Two saline washes of the incubation mixture on the filter were used to reduce the non-specific binding. Background was established by the addition of a final concentration of 10^{-4}M LRF. Filtration of whole cell preparations was carried out using only Whatman GF/c filters as aromatic, hydrophobic peptides adsorb to cellulose nitrate filters used previously in the assay of TRF binding sites (4, 5). Incubations were carried out at 0.2 ml final volume in Hepes buffer (8).

RESULTS AND DISCUSSION

Analogous to the binding kinetics of a number of peptide hormones to their cellular receptors (i.e., TRF (11), oxytocin (12) and insulin (13)), [^{3}H]LRF binds to two distinct sites (Figure 1a). One [^{3}H]LRF binding site has an affinity close to the determined biological activity constant of ca. 2 X 10^{-9}M LRF; the other has a much lower affinity (ca. 2 X 10^{-8}M LRF) but a higher capacity to bind [^{3}H]LRF. The role, if any, of a low affinity

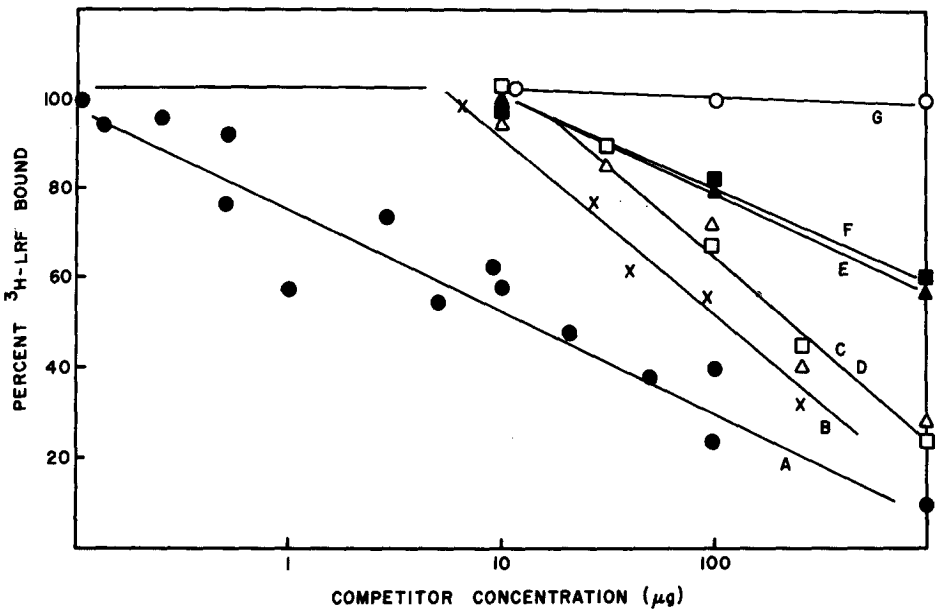

Figure 2. Competition curves of LRF and LRF analogues for the binding of [^{3}H-Pro^{9}]LRF to the low affinity, high capacity, non-specific binding site. Percent biological potency of each analogue is given in parentheses A. LRF (100) B. [OMe-Tyr^{5}]LRF (35) C. des-Arg^{8}-LRF (< 10^{-4}) D. des-<Glu-Ac^{1}-LRF (1) E. [Gly^{2}]LRF * F. des-His^{2}-LRF * G. TRF (< 10^{-5}) * antagonists (2).

site in the activation of the secretion rate of LH and FSH is unknown; due to its high capacity, its presence on the cells renders the examination of the second, high affinity, apparently physiological, receptor technically very difficult. In preliminary experiments examining the specificity of the low affinity site it was possible to show that although this site has the ability to recognize [^{3}H]LRF with some degree of preference, it does not show the same specificity characteristics as the physiological receptor (8). Although TRF does not compete with [^{3}H]LRF for binding, high activity analogues (such as [OMe-Tyr^{5}]LRF (35% potency of LRF) compete for [^{3}H]LRF binding with a lower than expected affinity and some essentially inactive LRF derivatives such as des-Arg^{8}-LRF (<.0001% potency of LRF) compete to an equal degree (1) for this high capacity site (Figure 2). These observations suggest that the dispersed pituitary cells possess a site (affinity constant 2 X 10^{-8}M LRF) for binding LRF related peptides, but that this site is not

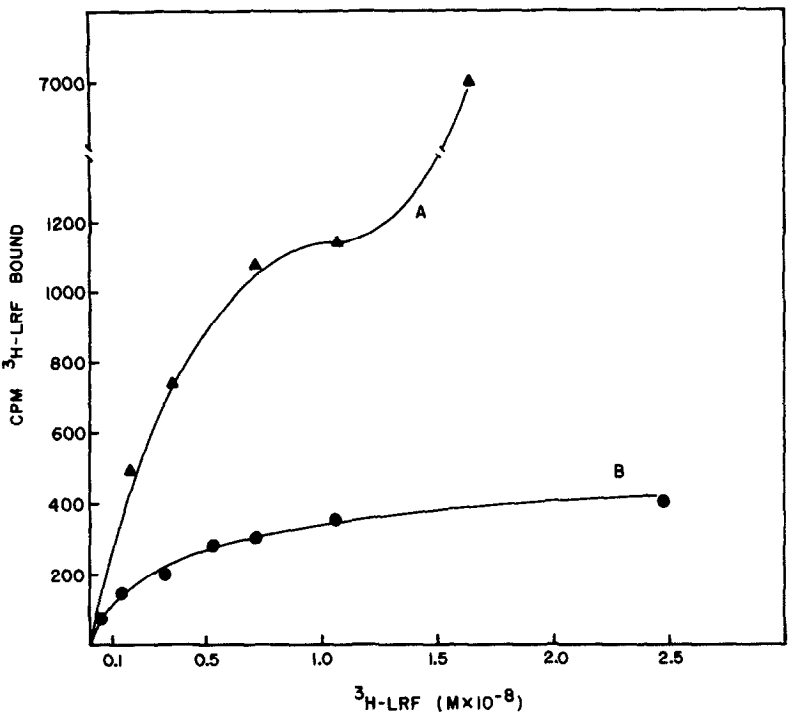

Figure 3. Binding curve of [^{3}H-Pro^9]LRF to the high affinity LRF binding site in the absence (A. ▲—▲) and presence (B. ●—●) of the inactive des-Arg^8-LRF analogue (1.5 mM).

the physiological LRF binding site as concluded from both its low LRF affinity and lack of specificity. This is in contrast with the observations reported for the low affinity site of [^{3}H]TRF binding in both normal and neoplastic pituitary cells which indicated slightly altered specificities but to a degree which was only demonstrable by strenuous analysis (11).

The [^{3}H]LRF binding site which has an affinity constant close to that obtained in secretion experiments was examined qualitatively by saturating the non-specific, low affinity site. The saturation was accomplished by the addition of excess des-Arg^8-LRF and then examining the competitive titration of the residual high affinity, [^{3}H]LRF binding by various LRF analogues. Figure 3 shows a binding curve of [^{3}H]LRF in the presence and absence of 1.5 mM des-Arg^8-LRF, demonstrating that the kinetic parameters of

Table 1. Competition for the binding of [^{3}H]LRF to normal rat pituitary cells in the presence of 1.5 mM des-Arg^8-LRF.

Treatment[b]	cpm [^{3}H]LRF[a] bound ± S.E.M.
None (21)	1007 ± 28
30 ng LRF (6)	820 ± 39
100 ng LRF (6)	799 ± 50
300 ng LRF (6)	807 ± 18
1 µg LRF (5)	651 ± 38
100 µg LRF (3)	611 ± 51
10 µg des-His^2-LRF (6)	758 ± 29
100 µg des-His^2-LRF (6)	688 ± 34
1 mg des-His^2-LRF (6)	662 ± 36
10 µg [Gly^2]LRF (3)	855 ± 80
100 µg [Gly^2]LRF (3)	756 ± 53
1 mg [Gly^2]LRF (3)	619 ± 21
100 ng [OMe-Tyr^5]LRF (3)	797 ± 100
1 µg [OMe-Tyr^5]LRF (3)	680 ± 53

[a]Final concentration of 1 X 10^{-8}M [^{3}H]LRF.

[b]( ) = number of replicates per treatment.

the binding to this high affinity [^{3}H]LRF binding site are unaffected by the presence of the inactive LRF analogue. Table 1 shows data on the competitive binding of a number of high and low activity LRF agonist analogues and also two antagonistic peptides. Although the marginal level of competible binding above background makes quantitation virtually impossible, concentration curves indicate competition for [^{3}H]LRF binding at levels of the same order of magnitude as the affinity constants ascertained biologically.

These data substantiate our conclusions (2) that both the agonistic and antagonistic LRF peptides manipulate the secretion rate of LH and FSH via common binding sites in competitive fashion.

CONCLUSIONS

The dispersed normal rat anterior pituitary cells used in these binding experiments possess two distinct binding sites for [^{3}H]LRF. One of the detected sites possesses the parameters of the physiological LRF receptor having an affinity constant of ca. 1 X 10^{-9}M LRF and a specificity for LRF analogues similar to the known biological activities of these analogues. The second site has no apparent relevance to the activities of the releasing factor having a lower affinity constant (2 X 10^{-8}M) and lacking an ability to distinguish between active and inactive LRF derivatives, but able to discern them from TRF.

The binding of [^{3}H]LRF to the high affinity site is competible by not only LRF agonists but also the two reported LRF antagonists at concentrations equivalent to their known inhibitory activities.

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REFERENCES

1. Vale, W., G. Grant and R. Guillemin, in Frontiers in Neuroendocrinology, 1973, L. Martini and W. Ganong (eds.), Oxford University Press, New York, 1973, in press.
2. Vale, W., G. Grant, J. Rivier, M. Monahan, M. Amoss, R. Blackwell, R. Burgus and R. Guillemin, Science, 176, 933, 1972.
3. McCann, S., in Frontiers in Neuroendocrinology, L. Martini and W. Ganong (eds.), Oxford University Press, New York, 1971, p. 209.
4. Grant, G., W. Vale and R. Guillemin, Biochem. Biophys. Res. Commun., 46, 28, 1972.
5. Labrie, F., N. Barden, G. Poirier and A. DeLean, Proc. Nat. Acad. Sci. USA, 69, 283, 1972.
6. Guillemin, R., R. Burgus and W. Vale, in Vitamins and Hormones, 29, P. Munson (ed.), Academic Press, New York, 1971, p. 1.
7. Grant, G. and W. Vale, Nature (New Biology), 237, 182, 1972.

8. Vale, W., G. Grant, M. Amoss, R. Blackwell and R. Guillemin, Endocrinology, 91, 562, 1972.
9. Rivier, J., M. Monahan, W. Vale, G. Grant, M. Amoss, R. Blackwell, R. Guillemin and R. Burgus, Chimia, 26, 300, 1972.
10. Monahan, M. and J. Rivier, Biochem. Biophys. Res. Commun., 48, 1100, 1972.
11. Grant, G., W. Vale and R. Guillemin, Endocrinology, submitted for publication.
12. Bockaert, J., M. Imbert, S. Jard and F. Morel, Molecular Pharm., 8, 230, 1972.
13. Freychet, P., in Proceedings of the IVth International Congress of Endocrinology, Excerpta Medica, Amsterdam, in press.