Vol. 95, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1980 Pages 256-262

LEYDIG CELL RECEPTORS FOR LUTEINIZING HORMONE RELEASING HORMONE AND ITS AGONISTS AND THEIR MODULATION BY ADMINISTRATION OR DEPRIVATION OF THE RELEASING HORMONE.

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Received May 2,1980

SUMMARY: Leydig cells isolated from adult rat testes bound ^{125}I-labelled luteinizing hormone releasing hormone (LHRH) agonist with high affinity (K_A=1.2 x 10^9M) and specificity. LHRH and the 3-9 and 4-9 fragments of LHRH agonist competed for binding sites with ^{125}I-LHRH agonist but with reduced affinities, whereas fragments of LHRH, and oxytocin and TRH were largely inactive. Somatostatin inhibited binding at high (10^{-4}M) concentrations but was inactive at 10^{-6}M and less. Pretreatment of rats for 7 days with 5 μg/day of LHRH agonist reduced binding of ^{125}I-LHRH agonist to Leydig cells in vitro by 25%, whilst inhibition of endogenous LHRH by antibodies for 7 days caused a 40% decrease.

It is clearly established that agonists of LHRH have drastic inhibitory effects on reproductive function in the male and female (1-3). In the male, Leydig cell function is impaired as evidenced by reductions in the number of LH-receptors and in steroidogenic responsiveness (2,4). These changes are identical to those observed after administration of high doses of LH or hCG (5), suggesting that the actions of the LHRH agonist are mediated by the release of pituitary LH. However, similar changes have also been reported recently following treatment of hypophysectomized rats with high doses of LHRH agonist (6,7). If the latter effect is specific, then Leydig cells should possess receptors for LHRH agonists. In this paper we demonstrate the existence of such receptors and show that their numbers can be modulated by alteration of the exposure of the Leydig cells in vivo to LHRH activity.

MATERIALS AND METHODS

Animals and Treatment: Testes from adult Sprague-Dawley rats were used to prepare isolated Leydig cells. In two experiments, the animals were pretreated. In the first, groups of 3 rats were injected subcutaneously daily

Abbreviations LHRH=luteinizing hormone releasing hormone ; LH=luteinizing hormone ; hCG=human chorionic gonadotrophin ; TRH=thyrotrophin releasing hormone ; HSA=human serum albumin ; BSA=bovine serum albumin.

0006-291X/80/130256-07$01.00/0

for 7 days with either saline (controls) or with 0.25 or 5 μg (D-Ser-t-bu^6, des-Gly-NH$_2^{10}$) LHRH ethylamide (LHRH agonist; Hoechst, U.K.), and were killed with ether 24h after their last injection. In the second experiment, endogenous LHRH was inhibited in groups of 3 rats for either 2 or 7 days by the injection of sheep antiserum to LHRH. The antiserum was raised to LHRH conjugated to HSA by carbodiimide (8), and rats received 2 ml antiserum intravenously on day 1 followed by a subcutaneous injection of 1 ml on day 2; control rats were similarly treated with serum from sheep immunized against HSA alone. The serum levels of testosterone in the above treated rats was determined by radioimmunoassay as desribed previously (4).

Hormones: LHRH agonist and its 3-9, 4-9 and 6-9 fragments, LHRH and its 1-6 and 3-10 fragments, TRH and somatostatin were all provided by courtesy of Dr. J. Sandow (Hoechst, A.G). Oxytocin was a gift from Sandoz Labs whilst 1-9 LHRH was supplied by courtesy of Dr. M. Hedlund (Abbott Labs).

Preparation of isolated Leydig cells and seminiferous tubule cells: Decapsulated testes in groups of 6 were teased slightly apart with forceps and placed into Krebs-Ringer bicarbonate solution (KRB; 1.5 ml/testis) containing 0.25 mg/ml collagenase (Type 1; Sigma). Testicular dispersion was effected by incubating in a shaking water bath for 15 min at 34 ^{o}C. The tissue was then diluted with KRB to 50 ml in a measuring cylinder and the seminiferous tubules allowed to settle. The supernatant was aspirated, centrifuged for 5 min at 350g and the precipitated Leydig cells resuspended in 0.025M Tris-HCl buffer (pH 7.5) containing 0.2% BSA (fraction V; Sigma) and 0.2 mg/ml bacitracin (Sigma). The resuspension volume was 2 ml/testis.

The discarded seminiferous tubules were washed 5 times with 50 ml 0.9% saline and lengths of individual tubules were selected and gently dispersed by repeatedly aspirating them into a 1 ml syringe. Cell clumps and undispersed material were removed by filtering through 3 layers of fine gauze and cells in the filtrate were precipitated by centrifuging for 5 min at 350g. The cells were resuspended in 0.025 M Tris-HCl containing 0.2% BSA and 0.2 mg/ml bacitracin.

Preparation of ^{125}I-LHRH agonist and ^{125}I-hCG: The LHRH agonist was labelled with ^{125}I using lactoperoxidase (9) and purified on a 40 x 1 cm column of Sephadex G25 fine (Pharmacia) which was eluted with 0.01N Acetic acid containing 0.1% BSA. This enabled clear separation of mono- and di-^{125}I-LHRH agonist (10, 11) as the former gave 3.2% specific binding to Leydig cells whereas the peak corresponding to di-^{125}I-LHRH agonist gave only 0.6% specific binding. The specific activity of the mono- ^{125}I-LHRH agonist which was used for all binding studies, was determined by self-displacement in the receptor assay and varied from 990-1150 mCi/mg. hCG (NIAMDD CR115) was labelled with ^{125}I to a specific activity of 52 mCi/mg as described previously (4) using the method of Miyachi *et al.* (12).

Measurement of the binding of ^{125}I-LHRH agonist and ^{125}I-hCG: Aliquots of 0.2 ml of the Leydig cell preparation were dispensed into 63 x 11mm polystyrene tubes, followed by unlabelled LHRH agonist or other hormones in 0.05 ml Tris-HCl buffer and finally by ^{125}I-LHRH agonist in 0.05ml Tris-HCl buffer. For most studies the incubation concentration of ^{125}I-LHRH agonist was 400pM (approx. 90,000 cpm) but in Scatchard analyses, concentrations of between 25 and 5200 pM were used. Non-specific binding was determined in the presence of 10^{-6}M unlabelled LHRH agonist. All incubations were performed in triplicate. Incubation was for 30 min at 21^{o}C and was terminated by placing tubes on ice and immediately diluting the incubate with 1 ml ice-cold Tris-HCl buffer. Tubes were then centrifuged for 10 min at 1000g and radioactivity in the pellet measured in a gamma counter.

As hormone treatment may affect the total yield, although not the proportion (approx. 30%), of Leydig cells obtained by collagenase dispersion of adult rat

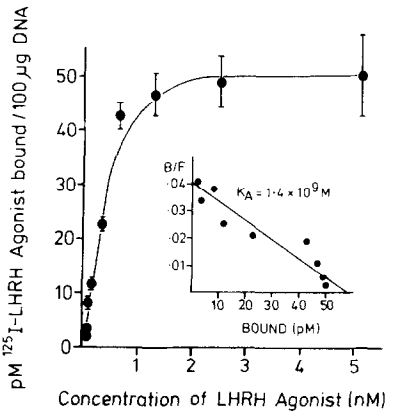

Fig. 1. Binding of ^{125}I-LHRH agonist to isolated Leydig cells at different concentrations of the radioligand. The inset shows the data as a Scatchard plot. Each point is the mean ± SD for triplicate incubations.

testes (13), the nucleated cell content of each Leydig cell preparation was determined by measuring the DNA content (14), and binding of ^{125}I-LHRH agonist has therefore been expressed as pM bound/100 µg DNA. Using the above conditions of incubation between 6.1 and 7.9% of the total ^{125}I-LHRH agonist added was bound per tube (which contained an average of 24 µg DNA), of which 48-54% was not displaceable by incubation with 10^{-6}M unlabelled LHRH agonist, and was taken to represent the non-specific binding.

To determine the cellular specificity of binding of ^{125}I-LHRH agonist, varying proportions of the Leydig cell and seminiferous tubule cell preparations were incubated together under the conditions described above. As a control, parallel incubations were performed using ^{125}I-hCG (20ng/ml) in place of ^{125}I-LHRH agonist as it is known that hCG binds exclusively to Leydig cells (15). The latter incubations were for 1 h at 34°C and other details were as described previously (13).

RESULTS

In preliminary experiments it was established that the binding of ^{125}I-LHRH agonist to Leydig cells reached equilibrium within 30 min at 21°C whilst 2-3h was required for equilibration of binding at 4°C. Binding was saturable and was to a single class of high affinity binding sites (Fig. 1), and in 4 experiments the mean (±SD) K_A of binding was 1.2 ± 0.3 x 10^9M. Binding was specific for LHRH agonist, its fragments and for LHRH, although there were marked differences in the binding affinity of these hormones (Fig. 2). Thus, LHRH was approximately 100 times less potent than the LHRH agonist in inhibiting binding whilst the potency of fragments of the LHRH agonist decreased with decrease in size so that the 3-9 fragment was 100 times less potent than the intact LHRH agonist

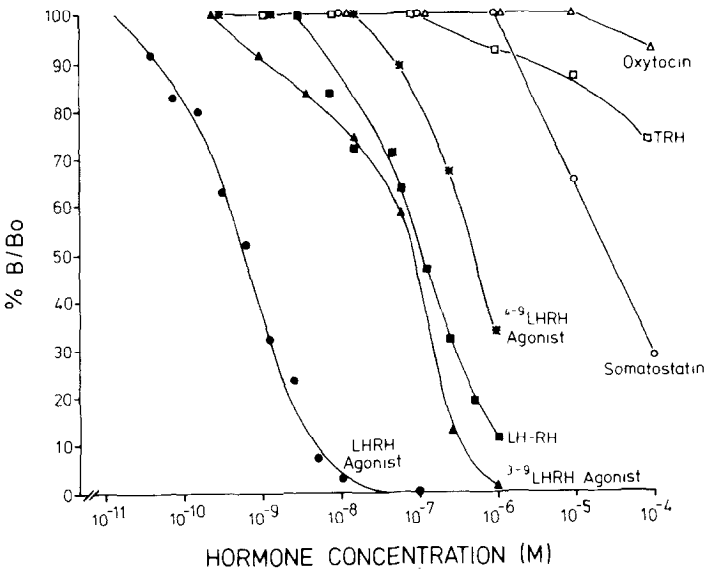

Fig. 2. Specificity of the binding of ^{125}I-LHRH agonist to isolated Leydig cells. Each point is the mean of triplicate incubations. Specificity was checked in a repeat experiment and essentially the same results obtained.

but was 10 times more potent than the 4-9 fragment (Fig. 2), whilst the 6-9 fragment was inactive at 10^{-6}M. The 1-6, 1-9 and 3-10 fragments of LHRH were completely inactive at concentrations up to 10^{-6}M. Of the other hormones tested, TRH and oxytocin showed only a small degree of binding activity and then only at very high concentrations whilst somatostatin inhibited 70% of binding at 10^{-4}M but was inactive at 10^{-6}M and less (Fig. 2).

With respect to the cellular location of receptors, the binding of ^{125}I-LHRH agonist decreased serially with decrease in the proportion of Leydig cells in the incubation medium (as indicated by the binding of ^{125}I-hCG) and binding to a 100% seminiferous tubule cell preparation was not significantly different ($P>0.2$) from zero (Fig. 3).

Leydig cells from rats pretreated for 7 days with either 0.25 or 5 µg/day of unlabelled LHRH agonist showed reduced binding of ^{125}I-LHRH agonist, with 10 and 25% reductions respectively (Fig. 4). As determined by 2-factor analysis of variance (with replication), both of these reductions were

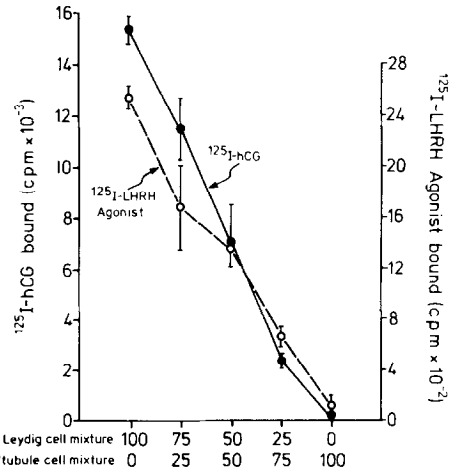

Fig. 3. Cellular specificity of the binding of ^{125}I-LHRH agonist. The proportion of Leydig cells was checked by the in vitro binding of ^{125}I-hCG. Each point is the mean ± SD for triplicate incubations.

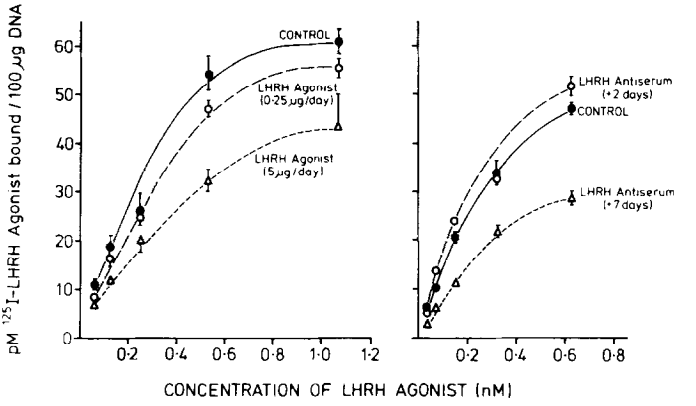

Fig. 4. Effect of treatment for 7 days with LHRH agonist (left) or injection of antiserum to LHRH for 2 or 7 days (right) on the binding of ^{125}I-LHRH agonist to isolated Leydig cells. Each point is the mean ± SD for triplicate incubations.

statistically significant ($P<0.05$ and $P<0.001$ respectively). There was no change in the affinity of binding. Both treatments reduced ($P<0.01$) the serum levels of testosterone (Controls: 9.5 ± 1.7 (SD) ng/ml; 0.25 µg agonist: 3.2 ± 0.1 ng/ml; 5 µg agonist: 3.0 ± 0.8 ng/ml).

Injection of rats with antiserum to LHRH for 2 days was without significant effect ($P>0.05$) on the binding of ^{125}I-LHRH agonist to Leydig cells whilst exposure to antibodies for 7 days reduced ($P< 0.001$) binding by nearly

40% (Fig. 4). Neither treatment affected the affinity of binding but both reduced the serum levels of testosterone (Controls: 3.9 ± 2.3 ng/ml; A/S 2 days: 0.8 ± 0.8 ng/ml; A/S 7 days 1.1 ± 0.1 ng/ml).

DISCUSSION

These results demonstrate that Leydig cells from the adult rat testis contain high affinity binding sites, which are specific for LHRH and its agonists, and appear to have similar characteristics to binding sites found on pituitary cell membranes (16) and those reported recently on rat luteal cells (17). It is evident that the binding sites are affected by the hormone environment as administration of unlabelled LHRH agonist or inhibition of endogenous LHRH by injection of antibodies (which in effect are entirely opposite treatments), both significantly reduced the number of LHRH agonist receptors within a week of treatment. Whether this change is related to the degree of exposure to LHRH activity is unknown but it is also noteworthy that both treatments reduced the serum levels of testosterone. Testosterone has been implicated in the regulation of pituitary responsiveness to LHRH (18), perhaps via effects on LHRH-receptors, raising the possibility of its importance in the regulation of gonadal LHRH agonist receptors.

In terms of the specificity of LHRH agonist-receptor interaction, the ability of the 3-9 and 4-9 fragments of LHRH agonist to competitively inhibit binding of the intact agonist (albeit with much reduced affinity), suggests that the first 2 or 3 amino acid sequences are not absolutely essential for binding. The ability of somatostatin to compete for LHRH agonist-binding sites at very high concentrations is unexplained, but it may be relevant that a recent report described LHRH-like activity of an analogue of somatostatin (19).

As yet the physiological function and importance of Leydig cell receptors for LHRH and its agonist is unknown, but it is presumably via these receptors that the reported (6, 7) direct effects of LHRH agonists on Leydig cell function occur. In view of the current interest in the antifertility effects of LHRH

agonists, a better understanding of the characteristics and modulation of gonadal receptors for these compounds must be of considerable fundamental importance.

Acknowledgements: We are grateful to Dr. J. Sandow and Dr. S. Dombey (Hoechst), and the NIAMDD, U.S.A. for gifts of hormones, to Dr. T.A. Bramley for DNA measurements and to Mrs M. Swaney for technical assistance.

REFERENCES

1. Johnson, E.S., Gendrick, R.L. & White, W.F. (1976) Fertil. Steril., 27, 853-860.
2. Labrie, F., Auclair, C., Cusan, L., Kelly, P.A., Pelletier, G. & Ferland, L. (1978) Int. J. Androl. Suppl., 2, 303-314.
3. Fraser, H.M., Laird, N.C. & Blakeley, D.M. (1980) Endocrinology, 106, 452-457.
4. Sharpe, R.M., Fraser, H.M. & Sandow, J. (1979). J. Endocr. 80, 249-257.
(1978) Int. J. Androl. Suppl. 2, 193-239.
6. Hsueh, A.J.W. & Erickson, G.F. (1979) Nature 281, 66-67.
7. Arimura, A., Serafini, P., Talbot, S. & Schally, A.V. (1979) Biochem. Biophys. Res. Commun. 90, 687-693.
8. Lincoln, G.A. & Fraser, H.M. (1979) Biol. Reprod. 21, 1239-1245.
9. Miyachi, Y., Chrambach, A., Mecklenburg, R & Lipsett, M.B. (1973) Endocrinology, 92, 1725-1730.
10. Sandow, J. & Konig, W. (1979). J. Endocr. 81, 175-182.
11. Copeland, K.C., Aubert, M.L., Rivier, J. & Sizonenko, P.C. (1979) Endocrinology, 104, 1504-1512.
12. Miyachi, Y., Vaitukaitis, J.L., Nieschlag, E. & Lipsett, M.B. (1972), J. Clin. Endocr. Metab. 34, 23-28.
13. Sharpe, R.M. & McNeilly, A.S. (1980) Molec. Cell. Endocr. (In Press)
14. Burton, K. (1956) Biochem. J. 62, 315-323.
15. Catt, K.J. & Dufau, M.L. (1976) Biol. Reprod. 14, 1-15.
16. Clayton, R.N., Shakespear, R.A., Duncan, J.A. & Marshall, J.C. (1979) Endocrinology, 104, 1484-1494.
17. Clayton, R.N., Harwood, J.P., & Catt, K.J., (1979) Nature, 282, 90-92.
18. Labrie, F., Drouin, J., Ferland, L., Lagace, L., Beaulieu, M., de Lean, A., Kelly, P.A., Caron, M.G. & Raymond, V. (1978) Recent Progr. Horm. Res. 34, 25-93.
19. Bex, F.J., Corbin, A., Sarantakis, D. & Lien, E. (1980) Nature, 284, 342-343.