Studies on Rat Ovarian Receptors for Lutropin (Luteinizing Hormone)

APPLICABILITY OF RADIOIMMUNOASSAY TO MEASURE LUTROPIN BOUND TO RECEPTORS

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Measurement of receptor-bound unlabelled physiologically active lutropin (luteinizing hormone, LH) was possible by a modified radioimmunoassay. The conventional radioimmunoassay conducted at 4°C was inadequate, whereas the modified assay performed at 37°C could measure receptor-bound lutropin. The radioimmunoassay at 37°C takes only 36 h for completion compared with 5–7 days at 4°C. The sensitivity and range of dose–response curves are, however, unaltered. The validity of the technique was established by a number of criteria.

A number of ovarian functions require the presence of lutropin (luteinizing hormone, LH) in plasma at critical concentrations. Correlation of concentrations of lutropin in the plasma with ovarian function has necessitated the study of the nature of lutropin receptors. Interaction with a membrane receptor of the target tissue has been implicated as the first event in the mechanism of action of many polypeptide hormones, including lutropin (Cuatrecasas, 1974). The majority of studies on receptors for lutropin have utilized radioiodinated lutropin or human chorionic gonadotropin (hCG) (Lee & Ryan, 1971; Kammerman *et al.*, 1972; Lee & Ryan, 1972; Rajaniemi & Vanha-Pertulla, 1972; Gospodarowicz, 1973). Although a variety of novel methods have been developed to iodinate lutropin with minimum damage to the hormone (Miyachi *et al.*, 1972; Redshaw & Lynch, 1974; Haour & Saxena, 1974), preparation of radioiodinated lutropin with high specific radioactivity and no loss of biological potency has inherent difficulties. Further, the very addition of a heavy element such as iodine can be considered to render the hormone unphysiological.

An alternative is the use of unlabelled lutropin to monitor receptor-bound hormone by an elevated-temperature radioimmunoassay (Moudgal *et al.*, 1971). A detailed investigation of the efficacy of this approach is described in the present study, with rat ovary as the model, where changes in lutropin receptors as a function of physiological status of the animal have been investigated.

Materials and Methods

Hormones and chemicals

Highly purified lutropin (2.5×NIH-LH-S1 in biological activity) used for receptor-binding studies was a kind gift from Professor H. Papkoff and Professor C. H. Li, Hormone Research Laboratories, San Francisco, CA, U.S.A. The following chemicals were purchased from BDH Chemicals, Poole, Dorset, U.K., and were of AnalaR grade. Chloramine-T, sodium metabisulphite, NaCl, Na₂HPO₄·12H₂O, NaH₂PO₄·2H₂O, MgCl₂, KCl, EDTA was a product of E. Merck, Darmstadt, Germany. Sigma Chemical Co., St. Louis, MO, U.S.A., supplied Tris and theophylline. Mercaptoethanol and gelatin were purchased from Koch–Light laboratories, Colnbrook, Bucks., U.K., and Difco Laboratories, Detroit, MI, U.S.A., respectively. Carrier-free Na¹²⁵I was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., through the Bhabha Atomic Research Centre, Bombay, India.

Animals

Albino rats of our Institute colony, originally derived from the Wistar strain, were maintained under a light/dark schedule of 14 h:10 h. They were fed on standard pellet diet (Hindustan Lever Products, Bombay, India) and given water *ad libitum*. Superovulated immature rats were prepared by subcutaneous administration of 50 i.u. of pregnant-mare-serum gonadotropin twice, 48 h apart, followed by 25 i.u. of

human chorionic gonadotropin given subcutaneously 56h after the second injection (Parlow, 1961). They were used 5 days after receiving the human chorionic gonadotropin injection.

Antisera

Production and characterization of rabbit antiserum to sheep lutropin was by procedures described by Madhwa Raj & Moudgal (1970). Goat antiserum to rabbit γ -globulin was used in the radioimmunoassay (Moudgal & Madhwa Raj, 1974).

Tissue incubations for receptor-binding studies

Rats under light ether anaesthesia were killed by cervical dislocation. Ovaries were immediately removed, freed of surrounding fat and cut into small pieces. All handling of tissue was done in a Petri dish containing 0.9% NaCl and kept on crushed ice. Chopped tissue (15–20mg) was then incubated for 1 h at 37°C in a Dubnoff metabolic shaker in 2ml of Krebs–Ringer bicarbonate buffer containing 0.2% glucose (Umbreit *et al.*, 1964). Wherever included, solutions of lutropin were added in small volumes (10–20 μ l) to the required concentration. At the end of the incubation, the flasks were removed and stored frozen until analysed by radioimmunoassay.

Processing of the tissue for radioimmunoassay

Tissue, after thawing, was taken out of the medium and homogenized in a loose-fitting all-glass homogenizer with 4ml of 0.05M-disodium phosphate/0.05M-EDTA (pH7.4)/0.9% NaCl buffer. The homogenate was centrifuged at 2000g for 15 min in a MSE 6L refrigerated centrifuge. The pellet was washed with 3 $\times$ 2ml of this buffer, by resuspension and centrifugation at 2000g for 15 min. At the end of the third wash, the pellet was resuspended in the buffer at 20mg/ml (on the basis of the weight of the starting material). Suitable samples were directly pipetted into the appropriate radioimmunoassay tubes.

Radioimmunoassay of receptor-bound lutropin

A homologous elevated-temperature radioimmunoassay for sheep lutropin was used to measure lutropin bound to receptors. Iodination of lutropin was carried out essentially by the method of Greenwood *et al.* (1963), except that it was carried out at 4°C.

The labelled lutropin was checked for immunological activity with various dilutions of the antiserum before being used for the assay. The radioimmunoassay was conducted at 37°C. The total incubation time was 36–40h. All dilutions of hormone and antiserum were with cold phosphate/EDTA/NaCl buffer. A sample (100 μ l) of the suitably diluted antiserum was incubated for 10–12h with standards (sheep lutropin) or the '2000g pellet' (from the incubated tissue). Suitable controls with similarly diluted normal rabbit serum and the 2000g pellet were included to correct for non-specific binding. Then 100 μ l of 125 I-labelled lutropin (20000 c.p.m.) was added to each of the tubes and incubation continued for 10–12h. Then sufficient goat antibody to rabbit γ -globulin was added to precipitate bound lutropin over 10–12h. The tubes were then counted for radioactivity, centrifuged (2000g for 15 min), the supernatants were siphoned off and the pellets counted for radioactivity. Counting of radioactivity was done in a Packard γ -ray spectrometer.

A conventional double-antibody radioimmunoassay using diluted (1:10000) antiserum was also conducted at 4°C for comparison.

Results and Discussion

The specific radioactivity of the iodinated lutropin was 30–40 μ Ci/ μ g. Whereas conventional radioimmunoassay performed at 4°C failed to measure any lutropin bound to ovarian receptors, that performed at 37°C was able to measure physiological amounts of lutropin bound to ovarian receptors (Table 1). The latter method, in addition, offered a distinct advantage in that the duration of assay was only 36–40h, com-

Table 1. Results of measurement of tissue-bound lutropin by radioimmunoassay performed at two different temperatures

Minced ovarian tissue (15–20mg) from superovulated immature rats was incubated with or without 5 μ g of lutropin/ml. Later the tissues were processed for radioimmunoassay as described in the Materials and Methods section. Two radioimmunoassays were conducted, one at 4°C and the other at 37°C, to measure receptor-bound lutropin. Results are average of duplicate determinations. —, Measurement not practicable.

Temperature of assay	Dilution of antiserum	Sensitivity of the assay (ng)	Amount of tissue needed for the assay	Lutropin bound (ng/mg of tissue)	
				Control (–lutropin)	Experimental (+lutropin)
4°C	1:10000	0.5	Does not measure	0.01	0.01
4°C	1:1000	200	100–200mg	—	—
37°C	1:10000	0.5	2–4mg	0.05	0.18

Fig. 1. Comparison of standard curves obtained for the radioimmunoassays performed at 4°C (●) and 37°C (○) by using antiserum diluted 1:10000

The condition of the assay at 37°C is described in the Materials and Methods section. The procedure for the assay at 4°C is according to conventional protocols (Greenwood *et al.*, 1963). The precision of measurement (standard deviation in terms of percentage binding) was 7–5% for doses from 0 to 0.5 ng and 3 to 1% for doses from 1 to 10 ng. The variation within the assay and between the assays were 5% and 10–15% respectively. The standard curves represent the average values obtained from many assays conducted over a period of many years.

pared with 5–7 days for radioimmunoassay at 4°C. Further, the assay at 37°C did not differ significantly from that at 4°C in sensitivity or range of the standard curve. Some characteristics of the assay at 37°C are given in Fig. 1. Although the variation between assays is within normal range, all samples where comparison had to be made were assayed in a single radioimmunoassay to minimize such variation.

Perhaps the relative ability of the assay at 37°C compared with that at 4°C to measure receptor-bound lutropin could be explained as being due to the following factors.

The equilibrium dissociation constant (K_d) values for the hormone–receptor and hormone–antibody interactions are both about 1×10^{-11} M. Assuming multivalency for both, it is the relative concentration of receptor and antibody that would drive the hormone towards one or the other. Thus an excess of antibody should be able to arrest hormone action. In fact lutropin-stimulated steroidogenesis was arrested by the addition *in vitro* of antiserum to lutropin (Moyle *et al.*, 1971; Behrman *et al.*, 1971). Whether this is achieved by physical pulling of lutropin off the receptor, or by creating a sink for lutropin in the medium, is debatable. At least from our own observations, even mere depletion of lutropin in the medium would cause the bound lutropin to dissociate (Muralidhar & Moudgal, 1976). The antibody could

do this more effectively. In the present paper we have shown that whereas three washes of the tissue (after treatment with lutropin) with normal rabbit serum diluted 1:1000 was unable to remove specifically bound lutropin *in toto*, a single wash with antiserum diluted 1:1000 was sufficient to remove all the lutropin (Fig. 2). We have in fact observed that it is possible to measure receptor-bound lutropin in a radioimmunoassay at 4°C by using antiserum diluted 1:1000 but not 1:10000. The use of the former in a routine radioimmunoassay is, however, precluded, as it is very insensitive. It has been possible to overcome the sensitivity problem by carrying out the assay at 37°C, but with antiserum diluted 1:10000.

Fig. 2 further demonstrates that negligible amounts of lutropin were removed from the tissue after the third wash with normal rabbit serum, and therefore in all subsequent experiments the number of washes was limited to three in order to remove all non-specifically bound lutropin. The addition of ¹²⁵I-labelled lutropin to the medium just before commencement of washing also showed that three washes are sufficient to remove all the lutropin present in the medium and that non-specifically bound to tissue (results not shown).

The validity of the assay for measuring specific binding was further assessed by testing the hormonal and tissue specificity of lutropin binding. Non-target tissues such as lung and uterus did not retain any

Fig. 2. Experiment to test the ability of anti-lutropin antiserum (●) and normal rabbit serum (○) to remove tissue-receptor-bound lutropin

Tissue incubated with 5 µg of lutropin/ml of the medium was homogenized and centrifuged. The 2000g pellet was washed once with 0.05 M-phosphate/0.05 M-EDTA/0.9% NaCl, pH 7.4. The subsequent washes were made with either lutropin antiserum or normal rabbit serum, each diluted 1:1000. The residual amount of lutropin bound to the tissue was measured after each wash. The amount of lutropin bound to the tissue at the initial point (before the first wash) has not been measured and is represented by the broken lines in the Figure.

Table 2. *Specificity of lutropin binding*

Tissue (10–15 mg) was incubated with (5 µg/ml) or without hormone. The tissue was later processed for the measurement of bound lutropin or follitropin by the elevated-temperature radioimmunoassay as described in the Materials and Methods section. Follitropin was measured in a homologous radioimmunoassay specific for sheep follitropin (N. R. Moudgal, K. Muralidhar, A. J. Rao & C. S. Sheela Rani, unpublished observations). The values are average of duplicate determinations.

Tissue	Hormone	Hormone bound (ng/mg of tissue)
Uterus	–Lutropin	Nil
	+Lutropin	Nil
Lung	–Lutropin	Nil
	+Lutropin	Nil
Corpora lutea	–Lutropin	Nil
	+Lutropin	4.1
	–Follitropin	Nil
	+Follitropin	0.05
Ovarian follicle	–Follitropin	Nil
	+Follitropin	19.0

measurable lutropin, compared with ovary under identical incubation conditions (Table 2). Further, sheep follitropin (follicle-stimulating hormone, FSH) bound only slightly to luteal tissue when compared with sheep lutropin, in conformity with its known site of action (Table 2).

Although the amount of lutropin added to the medium to ensure that enough be retained by the tissue to be detected by radioimmunoassay was higher than that used by other workers, the amounts of lutropin bound per mg of tissue is still in the range obtained by Lee & Ryan (1971) using ¹²⁵I-labelled lutropin. This further stresses that radioimmunoassay can measure specifically bound lutropin in the physiological range. Most workers using ¹²⁵I-labelled lutropin (or human chorionic gonadotropin) differ from us by using either single cells (Kammerman *et al.*, 1972), thin sections (Lee & Ryan, 1971), homogenate (Lee & Ryan, 1972) or enriched plasma-membrane fractions (Gospodarowicz, 1973), where accessibility of the receptor to the hormone is not a problem. The sensitivity of the radioactive counting again permits use of small amounts of tissue and hormone.

An additional support for the validity of the present approach has been our observation that the

concentration of lutropin required to be present in the medium to detect binding and to give a response is the same (Moudgal *et al.*, 1971; Muralidhar & Moudgal, 1976).

In conclusion, the validity of the modified elevated-temperature radioimmunoassay conducted at 37°C in measuring physiological amounts of unlabelled lutropin bound to receptor has been established.

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