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Studies on Rat Ovarian Receptors for Lutropin (Luteinizing Hormone)

FACTORS INFLUENCING BINDING AND RESPONSE

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The interaction of rat ovarian receptors with lutropin (luteinizing hormone, LH) *in vitro* was rapid and reversible. The degree of binding was saturable and susceptible to changes in the concentration of lutropin in the medium. The concentration of lutropin receptors in the ovary increases during the natural pubertal period and also in immature rats given pregnant-mare-serum gonadotropin and human choriogonadotropin. In the latter case, the increase in lutropin receptor, after injection of pregnant-mare-serum gonadotropin alone, could be detected only if the ovaries are freed of the bound gonadotropin before exposure to lutropin. The concentration of lutropin receptors was higher in the luteal compartment of the ovary than in the non-luteal parts and increased slightly in aged corpora lutea. Correlation between binding of lutropin to the ovary and the ovarian response to lutropin in terms of cyclic AMP production was found only in prepubertal rat ovaries and in young corpora lutea and not in aged corpora lutea, suggesting the non-equivalence of binding *in vitro* and ovarian response.

In the preceding paper (Muralidhar & Moudgal, 1976) we described the use of an elevated-temperature radioimmunoassay to monitor unlabelled lutropin (luteinizing hormone, LH) bound to ovarian receptors. Here we describe investigations on the degree of binding of lutropin as a function of external concentration of lutropin and as a function of known changes in the physiological status of the ovary. Studies on the correlation of binding and response in ovaries from rats under different physiological states are also presented. A preliminary account of the results has been presented (Moudgal & Muralidhar, 1974).

Materials and Methods

All materials and procedures were described in the preceding paper (Muralidhar & Moudgal, 1976).

Radioimmunoassay of rat serum lutropin

Rat serum lutropin was measured by a heterologous system that uses rabbit antiserum to sheep lutropin and the β-subunit of sheep lutropin as label. Sheep lutropin was used as standard. Details of the assay are the same as those described for the radioimmunoassay of receptor-bound lutropin (Muralidhar & Moudgal, 1976).

Tissue incubation for cyclic AMP studies

The conditions of incubation were the same as those described for lutropin-binding studies except that 1 ml of Krebs–Ringer bicarbonate buffer containing 0.2% glucose (Umbreit *et al.*, 1964) and 8 mM-theophylline was used. The duration of incubation was 20 min and at the end of the incubation the tubes were immersed in a boiling-water bath for 10 min. The medium after centrifugation was used directly for cyclic AMP assay. The tissue was extracted for cyclic AMP as described below.

Measurement of cyclic AMP

The cyclic AMP in the tissue was extracted by the procedure of Ebadi *et al.* (1970), except that potassium aluminium sulphate was used instead of $ZnSO_4$, as this gave better recoveries. The cyclic AMP in the tissue extract and in the medium was assayed by the competitive binding assay essentially as described by Brown *et al.* (1971), except that Millipore filters (HAWP, 0.45 μm pore size) were used for separation of bound and free cyclic AMP.

Results

Nature of lutropin-receptor interaction

The stability and flexibility of lutropin-receptor interaction was studied by using two experimental

models involving the ovarian tissue. In the first model the ratio of lutropin present in the tissue and in the medium was kept unaltered. In the second model a deliberate attempt was made to alter the above ratio. Ovarian tissue from superovulated immature rats was incubated with 5μg of lutropin/ml of the medium under standard conditions as described previously (Muralidhar & Moudgal, 1976). The tissue was later homogenized and washed with phosphate/EDTA/NaCl buffer. The 2000g pellet obtained at the end of the third wash constituted an enriched lutropin-receptor preparation and hereafter is referred to as 'pellet'. This was resuspended in the above buffer (5mg/ml) and incubated at 25°C for 2h with occasional shaking. Analysis of the lutropin content

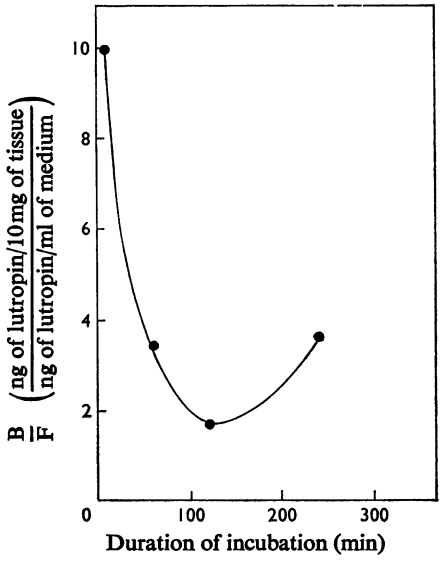

Fig. 1. *Flexibility of lutropin-receptor interaction in response to changes in concentration of lutropin in the medium*

Results are given of measurement of lutropin in the medium and tissue in an experiment where the equilibrium of lutropin-receptor interaction was deliberately disturbed. Ovarian tissue (16mg) was suspended in 3.2ml of the medium. At stipulated time-intervals, 0.3ml of the suspension was taken out followed by centrifugation (2000g for 15min) of the incubation mixture and taking out of 0.3ml of the medium alone. The amounts of tissue at each stipulated time-interval were therefore 16, 14.5, 12.8 and 10.9mg. The volumes of the medium at the respective time-intervals were 3.2, 2.6, 2.0 and 1.4ml. The amount of lutropin in the tissue and medium was measured separately by radioimmunoassay. Results are expressed as B/F ratio at each time-interval, where B is the amount of lutropin present in 10mg of tissue and F is the amount of lutropin present in 1ml of medium. For further details see the text.

in the medium and pellet was made at stipulated time-intervals. It was found that as long as the removal of samples for assay of lutropin did not disturb the equilibrium (by taking equal amounts of tissue and medium), the concentration of lutropin in the medium and tissue did not change. However, if equilibrium was disturbed by taking unequal amounts of tissue and medium (1 part of pellet and 2 parts of medium), the tissue released lutropin into the medium and this release was more than the amount of lutropin lost by removal of the additional portion of the medium. After the third sampling, the release of lutropin apparently caused the system to revert to an equilibrium where more lutropin was present in the tissue than at the previous sampling point (Fig. 1). This illustrates the highly reversible and flexible nature of the lutropin-receptor interaction.

The rat ovarian receptors are saturable with lutropin, as would be expected of specific receptors. The shape of the dose–response curve suggested a positive co-operative phenomenon (Fig. 2*a*).

Changes in lutropin-binding potential as a consequence of known changes in ovarian functionality

From the lutropin-binding curves plotted for different days of maturation, it is evident that the receptor concentration increases with maturation, reaching adult pattern by day 50. Coincident with the increase in lutropin receptor concentration there appeared to be a decline in serum lutropin concentration. Thus from a concentration of 1.3±0.2ng/ml seen on day 20 the value had fallen to 0.55±0.08ng/ml by day 50. These values were obtained by measuring the concentration of lutropin in groups of rats (seven each) killed on days 20, 30, 40, 50 and 60 of age. This tendency of lutropin concentrations in serum to decline to basal value before pubertal changes agrees well with the results of Goldman *et al.* (1971).

In immature rats primed with pregnant-mare-serum gonadotropin to simulate natural maturation, a significant increase (threefold) in the concentration of lutropin receptors in the ovary was observed after injection of human choriogonadotropin into the rats (Fig. 3).

The absence of increase in lutropin receptors after injection of pregnant-mare-serum gonadotropin alone could be due to masking of lutropin receptors by the gonadotropin, as the latter is known to possess both follitropin and lutropin activity, and has a long plasma half-life. To test this, ovaries from rats were incubated *in vitro* in an excess of diluted (1:1000) antisera to sheep follitropin and lutropin for 15min at 4°C. Subsequently they were washed free of antisera with 0.9% NaCl and incubated with lutropin under standard conditions. Table 1 shows that there is a clear increase in lutropin-receptor concentration.

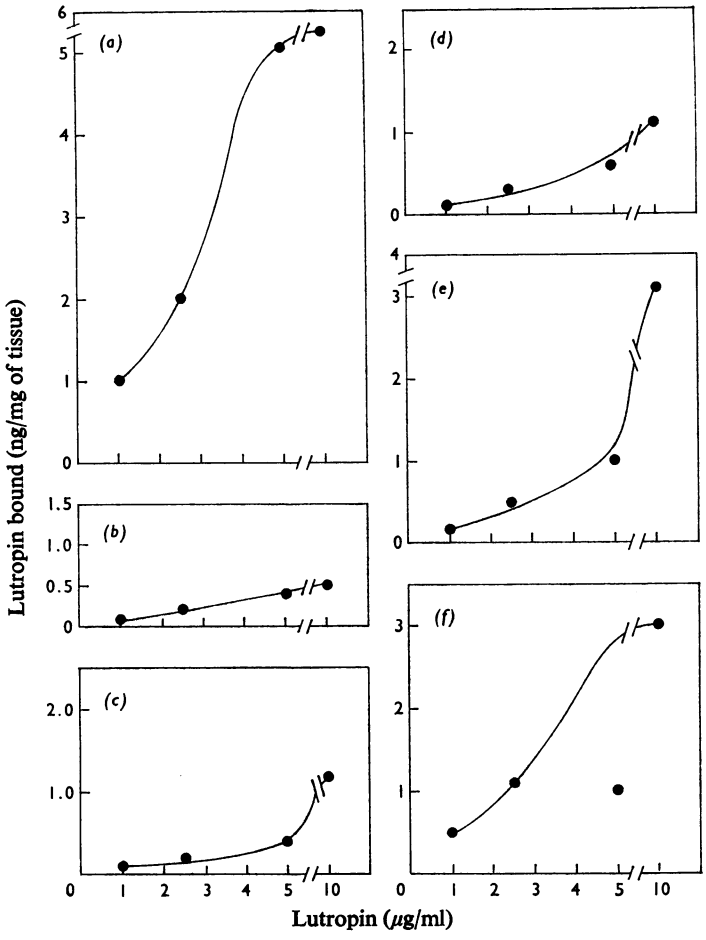

Fig. 2. *Pattern of dose–response curves for lutropin binding in vitro to ovaries from rats of 20–60 days of age and corpora lutea of adult cycling dioestrus rats*

Ovaries from 20- (*b*), 30- (*c*), 40- (*d*), 50- (*e*) and 60- (*f*) day-old rats or corpora lutea from adult cycling dioestrus rats (*a*) were incubated with graded amounts of lutropin for 1 h at 37°C and the bound lutropin was measured by radioimmunoassay. At each point a group of three rats were used. Incubations were done in duplicate. Results are the averages of duplicate determinations.

Corpora lutea of pregnant rats undergo changes in size and function during the progress of gestation and thus provide a good model to investigate the changes in the lutropin binding during luteal aging. Fig. 4 shows that during the progress of gestation there is an increase in the receptor content but not an increase in its concentration.

Studies demonstrating the dissociation of binding of lutropin from tissue response

The observed pattern of lutropin binding does not seem to parallel the decline in progesterone-synthesizing capacity seen in corpora lutea undergoing aging. We have therefore tried to correlate here the degree of binding of lutropin to its response measured in terms of cyclic AMP production.

The degree of binding of lutropin to immature rat ovaries was very low at any dose of lutropin, in contrast with its response in terms of cyclic AMP (Fig. 5*a*). Cyclic AMP increased in both the tissue and the medium. In contrast with the immature rat ovaries, which consist predominantly of interstitial tissue, in the heavily luteinized ovaries of rats treated with pregnant-mare-serum gonado-

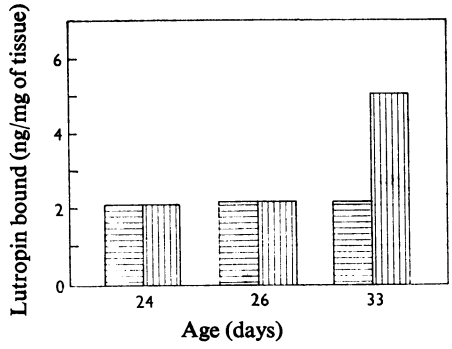

Fig. 3. *Changes in the binding potential in vitro of ovaries of immature rats after injections of pregnant-mare-serum gonadotropin and human choriogonadotropin*

Ovaries of three rats were pooled for each determination. The incubation medium (2ml) contained 5μg of lutropin/ml, the bound lutropin was measured by radioimmunoassay as described in the Materials and Methods section. Except for rats of group I, which received pregnant-mare-serum gonadotropin on day 22 (day 0), all rats received two injections of pregnant-mare-serum gonadotropin on days 22 and 24. Rats of group III received an ovulating dose of human choriogonadotropin on day 26. Binding of lutropin *in vitro* was tested for groups I, II and III on days 24, 26 and 33 of age respectively. Control rats (▤) received 0.9% NaCl and experimental rats (▥) received human choriogonadotropin and/or pregnant-mare-serum gonadotropin. Values are average of duplicate determinations.

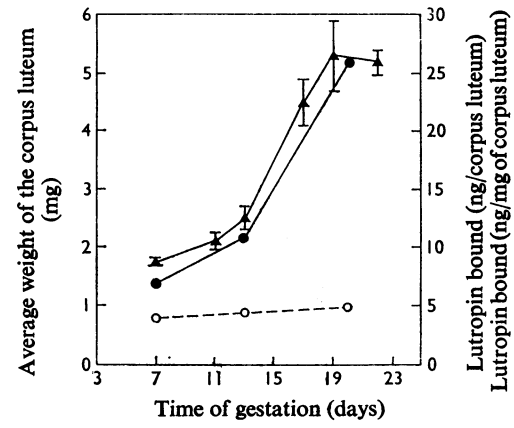

Fig. 4. *Correlation of specific binding of lutropin in vitro with increase in luteal weight* (▲) *during gestation in the rat*

Groups of three rats each were killed on specified days of gestation and corpora lutea were weighed and incubated with lutropin (5μg/ml). The bound lutropin was measured by radioimmunoassay per whole corpus luteum (●) or per mg of corpus luteum (○). Values are mean of three determinations. The weights of corpora lutea given above were obtained from data pooled from several experiments involving more than 10 rats at each specified day of gestation. Bars represent ±S.D.

Table 1. *Effect of a single injection of pregnant-mare-serum gonadotropin to immature rats on subsequent lutropin-binding potential in vitro*

Pregnant-mare-serum gonadotropin (50i.u. in 0.2ml of 0.9% NaCl) was administered subcutaneously to immature 22-day-old rats, and 48h later they were killed, ovarian follicles were dissected out and washed with a large excess of 1:1000-diluted antisera to follitropin and lutropin. The tissue was then washed free of antisera and incubated with lutropin (2.5μg/ml). Ovaries of corresponding NaCl-injected control rats were similarly processed. The amount of lutropin bound was measured by a specific radioimmunoassay. For details of procedure see the text. Each determination was made on ovaries from five rats; values are means±S.E.M.

Treatment *in vivo*	Hormone *in vitro*	Lutropin bound (ng/mg of tissue)
NaCl (0.9%)	−LH	0.1 ±0.03
	+LH	0.17 ±0.04
Pregnant-mare-serum gonadotropin	−LH	0.075±0.01
	+LH	0.30 ±0.06*

* Significantly different from the value for NaCl-treated rat ovaries incubated with lutropin ($P<0.05$).

tropin and human choriogonadotropin (Fig. 5*b*) and in the corpora lutea of dioestrus rats (Fig. 5*c*), the response to lutropin had declined considerably, although the binding of lutropin was more than that in immature rat ovaries. Both the release of cyclic AMP into the medium and the increase in the tissue concentration of cyclic AMP were therefore meagre.

In pregnant rats, net increase in cyclic AMP was found only in the case of corpora lutea of 8 day pregnant rats (Fig. 5*d*). In spite of increased binding of lutropin, the aged corpora lutea of 20 day pregnant rats and the post-partum rats (removed within 24h of parturition) did not respond to lutropin (Figs. 5*e*,5*f*). On the contrary, there was a significant decrease in the total cyclic AMP concentration. Mason *et al.* (1973), in a study on rat luteal response to lutropin during gestation, noted that except on day 6, where a marginal response was observed, there was no increase in cyclic AMP on the other days. In their study, however, response was not correlated with binding.

Another instance where response was absent in spite of good binding was in the corpora lutea of pregnant rats that were deliberately deprived of lutropin support for three consecutive days (days 5, 6 and 7) by injecting lutropin antiserum. Although such a treatment left the binding potential un-

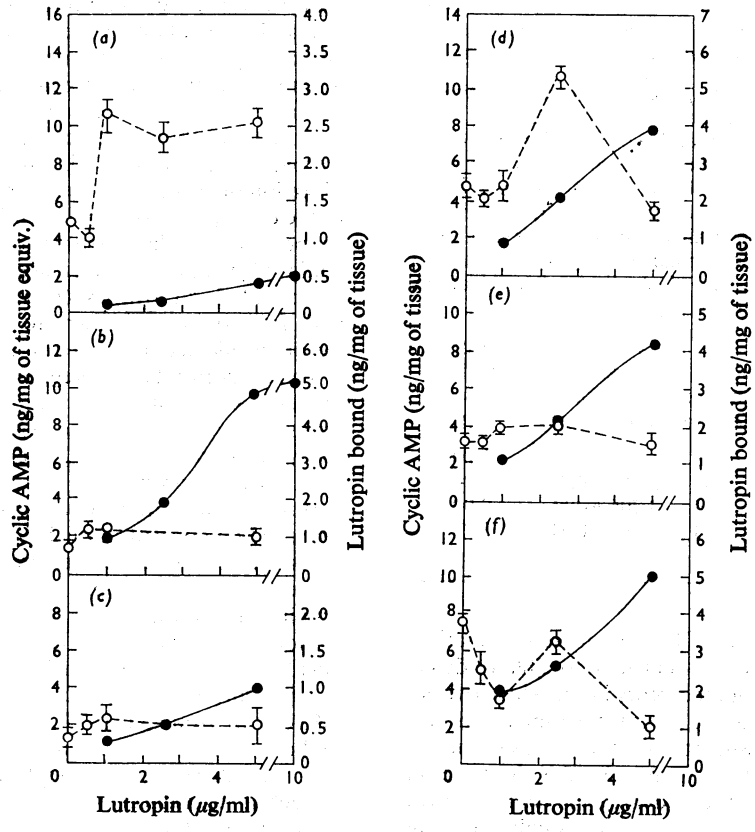

Fig. 5. *Comparison of the dose–response curves for binding of lutropin and response to lutropin in functionally different ovarian types*

Only the total cyclic AMP (tissue plus medium) is indicated in the Figure. Ovaries from immature rats (*a*) or corpora lutea from superovulated immature rats (*b*), adult cycling dioestrus rats (*c*), pregnant rats on day 8 (*d*) and day 20 (*e*) of gestation and from post-parturient rats on day 1 of lactation (*f*) were incubated with lutropin over the dose range of 0–5 μg/ml and the degree of lutropin binding (●) and degree of response to lutropin (cyclic AMP production, ○) was measured. Procedures for measurement of tissue-bound lutropin and for cyclic AMP measurement are given in the Materials and Methods section. The values for cyclic AMP are average of four determinations. The bars on the points refer to S.D. The values for lutropin binding are average of duplicate determinations. For each determination a group of three rats was used.

impaired, it abolished the potential of the luteal tissue to respond to lutropin in terms of cyclic AMP production (Table 2).

Discussion

By using porcine granulosa cells in culture, Channing & Kammerman (1973) concluded that the binding of ^{125}I-labelled human choriogonadotropin was tight, as it could not be completely displaced by unlabelled hormone. According to Paton (1961), the degree of response is directly proportional to the rate of association and dissociation of the bound drug/hormone. In confirmation of this is our finding that the binding of lutropin is not 'tight' and that its receptors possess properties of regulating the amount of lutropin bound. Under conditions *in vivo* the plasma concentration of lutropin does fluctuate and the ability of receptors to respond to such fluctuations is revealed in the present study.

The increase in lutropin receptors during pubertal changes perhaps reflects the changes in the functional status of the ovary. During puberty, the proportion of the follicular compartment increases and this is followed by an increase in the specific binding of lutropin to the ovary, implying that the follicular compartment has more receptors to lutropin than the interstitial tissue. Zeleznik *et al.* (1974) detected an increase in lutropin receptors in ovaries from immature rats after multiple injections of follitropin

Table 2. *Effect of treatment of pregnant rats with lutropin antiserum on days 5, 6 and 7 of gestation on the binding of lutropin in vitro and response in corpora lutea on day 8*

Pregnant rats (three each) were given lutropin antiserum (0.50ml/rat) or normal rabbit serum (0.5ml/rat) subcutaneously on days 5, 6 and 7 of gestation. On day 8, the corpora lutea were taken out, processed as usual and tested for both lutropin binding and response to the addition *in vitro* of 2.5μg of lutropin/ml. Values for cyclic AMP refer to total cyclic AMP concentrations (tissue plus medium) and are given as mean±S.D. Values for lutropin binding are the average of duplicate determinations. Results are average of two experiments for both.

Group	Hormone *in vitro*	Cyclic AMP (ng/mg of tissue equivalent)	Lutropin bound/mg of tissue (ng)
Normal rabbit serum treated	−LH	1.93 ±0.27	0.00
	+LH	3.76 ±0.23	2.20
Lutropin antiserum treated	−LH	3.206±0.56	0.00
	+LH	3.26 ±0.31	2.25

only when granulosa cells were used in isolation. The present study shows that it is possible to detect an increase in lutropin receptors in the whole ovaries as well, provided that the tissue was washed with antiserum to remove tissue-bound endogenous hormone. The role of other hormones in this increase in lutropin-receptor content during follicular growth is not clear at the moment, although work in hamsters indicates that in the total absence of lutropin, follitropin is unable to promote follicular maturation (C. S. Sheela Rani & N. R. Moudgal, unpublished work).

The absence of correlation between binding of lutropin to ovaries and well-known responses to lutropin, such as increase in cyclic AMP production and steroidogenesis under different physiological states, has been shown. For example, though the corpus luteum of the oestrous cycle (Macdonald, 1969) and the heavily luteinized ovaries (Armstrong, 1968) make steroids in response to lutropin, the present study demonstrates that the degree of increase in cyclic AMP in response to lutropin was only marginal, although the degree of binding was very good. In contrast, the day-20 immature rat ovary bound very little lutropin and produced large amounts of cyclic AMP. However, it is known to produce very little steroid in response to lutropin when compared with luteinized rat ovary (Mason, 1970).

Cyclic AMP is a key intermediate in the chain of events that follows lutropin binding, leading to enhanced steroid output (Marsh, 1969), and ovulation (H. Lipner, V. R. Mukku & N. R. Moudgal, unpublished work). The present study indicates that the correlation between lutropin binding and activation of adenylate cyclase is high in the ovaries of day-20 immature rats compared with others. The reasons for the low steroidogenic activity in these immature rat ovaries could be either insufficiency in the availability of cyclic AMP to activate steroidogenic enzymes (if these are present) or diversion of cyclic AMP to other unknown functions of the ovary, such as protein synthesis. An additional support for the last assumption is that follitropin could also cause an increase in cyclic AMP in pre-pubertal rat ovaries (Lamprecht *et al.*, 1973) and follitropin is known to be devoid of any steroidogenic activity (Lostroh & Johnson, 1966).

The corpora lutea of pregnancy, as they age, presented a situation where binding and response in terms of increase in cyclic AMP become progressively uncoupled. In fact, the pregnancy corpora lutea, which continue to be present in the postparturient rat, exhibited a significant decrease in cyclic AMP concentration in response to lutropin. It is also not clear at present whether high concentrations of lutropin induce phosphodiesterase activity in non-functional regressing corpora lutea. Although the loss of cyclic AMP response during luteal aging agrees well with the loss of other functions in aged corpora lutea, such as steroidogenesis, etc. (Mukku & Moudgal, 1975), the binding of lutropin to these corpora lutea is puzzling. It could mean loss of a 'transducer'. There could be a progressive loss of such a 'transducer' with aging of corpora lutea. However, whether the binding would be observed under conditions *in vivo* in the presence of other competing receptor sources, such as follicles, is open to question. Ahren *et al.* (1974), working with another model for aging corpora lutea, namely pregnant-mare-serum gonadotropin-primed immature rats made pseudopregnant with lutropin, also noted a progressive loss of response to lutropin in aged corpora lutea, but no studies correlating this to lutropin binding were performed. Our results, however, indicate that binding *in vitro* and response are not necessarily equivalent.

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