Ability of Human Chorionic Gonadotropin β-Subunit to Inhibit the Steroidogenic Response to Lutropin

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Ability of the β-subunit of human chorionic gonadotropin to inhibit the response to lutropin (luteinizing hormone, LH) was tested in the immature rat ovarian system and pregnant-mare-serum-gonadotropin-primed rat ovarian system with progesterone production being used as the response. Human chorionic gonadotropin β-subunit was found to inhibit human and ovine lutropin-stimulated progesterone production. At a constant dose of lutropin, inhibition was dependent on the concentration of β-subunit. When concentration of the β-subunit was kept constant at $5.0\,\mu g/ml$ and the concentration of lutropin was varied, the inhibition was maximum at the saturating concentration of the native hormone. The α-subunit of the human chorionic gonadotropin did not inhibit the response to lutropin. The lutropin/β-subunit ratio required to produce an inhibition of response was much lower than that required to bring about an observable inhibition of binding.

Ever since the subunits of gonadotropins became available for studies, attempts have been made to assess their biological activity. Although it is generally accepted that the subunits do not have any intrinsic biological activity (Catt *et al.*, 1973; Lee & Ryan, 1973), reports claiming such activity have appeared (Rao, 1973*a,b*; Yang *et al.*, 1972, 1973). It is also generally accepted, based on the binding of radio-iodinated hormones to isolated receptor preparations, that the subunits of gonadotropins do not bind to these receptors (Papaionannou & Gospodarowicz, 1975). Muralidhar & Moudgal (1976*a,b,c*), using un-labelled hormone and a modified radioimmunoassay procedure, have, however, shown that the β-subunit of ovine lutropin does bind specifically to rat ovarian lutropin receptors This was established by (*a*) determination of actual binding of β-subunit to lutropin receptors, (*b*) observing inhibition of binding of un-labelled intact human chorionic gonadotropin to the ovarian tissue and, finally, (*c*) observing an inhibition of response to ovine lutropin in terms of cyclic AMP production, although the subunit by itself was totally inactive.

In the present study we have investigated the ability of the β-subunit of human chorionic gonadotropin to inhibit a more specific and sensitive response to lutropin, such as stimulation of progesterone production in the rat ovary.

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Materials and Methods

Immature female albino rats (28–30 days old) of this Institute's colony (derived from the Wistar strain), with or without priming with 15i.u. of pregnant-mare-serum gonadotropin, were used in the present study. Human lutropin and ovine lutropin were gifts from Professor H. Papkoff, San Francisco, CA, U.S.A., and ovine and human lutropin given by Dr. M. R. Sairam, Montreal, Canada, were also used. The α- and the β-subunits of human chorionic gonadotropin (CR 119) used were kind gifts from Dr. R. Canfield, New York, NY, U.S.A. $[1,2,6,7-{}^3H]$-Progesterone was purchased from New England Nuclear, U.S.A. Carrier-free ^{125}I as NaI was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals used were of analytical grade.

Animals were killed by cervical dislocation. Ovaries pooled from several animals were incubated in Krebs–Ringer bicarbonate medium containing 20mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes buffer) and 0.1% bovine serum albumin (pH 7.4) at 37°C for 30min. About 15mg of tissue was suspended in 1 ml of fresh medium in a flask and incubated with or without human chorionic gonadotropin β-subunit at 37°C for 30min. Human or ovine lutropin was then added, the flasks were oxygenated, tightly stoppered and incubation was continued for an additional 2h. Progesterone secreted into the medium was determined by a specific radioimmuno-

assay (Mukku & Moudgal, 1975). The tissue-bound lutropin was determined by the method described in detail by Muralidhar & Moudgal (1976a).

Unless otherwise stated, α-subunit and β-subunit refer to human chorionic gonadotropin.

Results

Dose-dependent response to human lutropin and human chorionic gonadotropin β-subunit

Typical dose–response curves for these hormones, from both the immature and the pregnant-mare-serum-gonadotropin-primed rat ovarian systems, are shown in Fig. 1: the β-subunit at all doses used had virtually no activity. In this system 5–10 μg of the β-subunit showed an activity equivalent to less than 10 ng of human lutropin. Morgan et al. (1974) have reported that this preparation of human chorionic gonadotropin β-subunit in the rat ventral prostate-weight assay has an activity equivalent to 5 i.u. of human chorionic gonadotropin/mg.

Effect of β-subunit on the response to human and ovine lutropin

Table 1 shows the effectiveness of human chorionic gonadotropin β-subunit in inhibiting the response to lutropin in both the immature ovarian and the primed rat ovarian systems. The results show that the β-subunit (5 μg/ml) markedly inhibited the stimulation of progesterone production observed with saturating concentrations of human and ovine lutropin. Generally, the immature ovarian systems showed a higher percentage of inhibition.

Effect of changing the concentrations of human chorionic gonadotropin β-subunit and human or ovine lutropin on the response to lutropin

Table 2 shows the results of several independent experiments on the effect of change in the concentration of β-subunit on the response to human or ovine lutropin. It is evident that inhibition observed is reproducible and is dose-dependent.

In the pregnant-mare-serum-gonadotropin-primed rat ovarian system, marked inhibition of response by β-subunit (5 μg/ml) was observed only at saturating concentration of ovine lutropin. At the subsaturating concentration (0.5 μg/ml) there was a slight increase in response (Fig. 2).

Correlation between binding and response

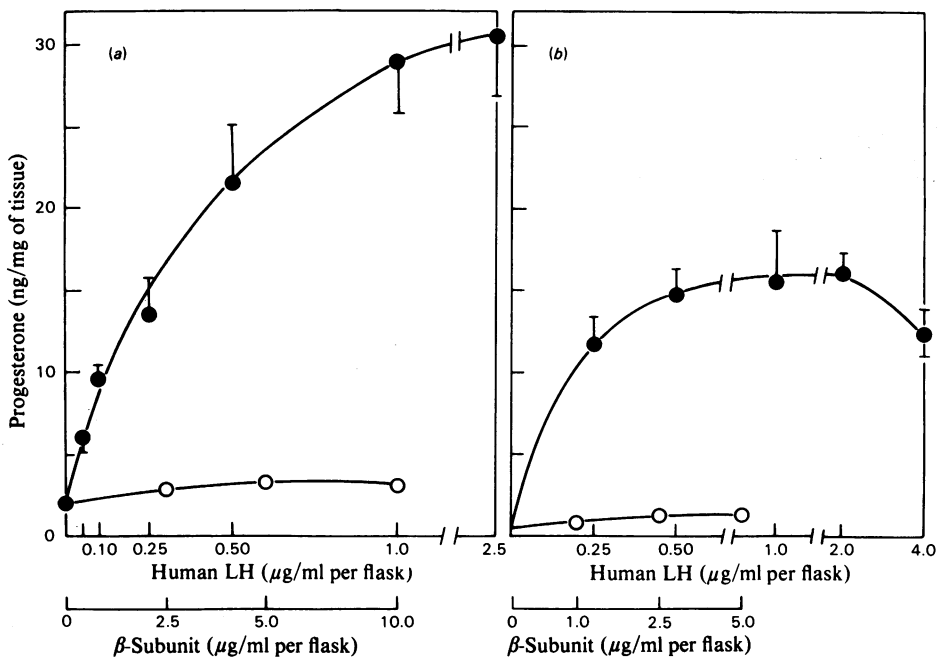Ovaries pooled from pregnant-mare-serum-gonadotropin-primed rats were incubated with in-

Fig. 1. Dose–response curves for human lutropin (LH) and human chorionic gonadotropin β-subunit in (a) the pregnant-mare-serum-gonadotropin-primed ovarian system and (b) the immature ovarian system

Minces of pooled ovarian tissue were incubated at 37°C with human LH or the β-subunit in 1 ml of Krebs–Ringer bicarbonate medium containing Hepes and bovine serum albumin (pH 7.4) for 2 h. Progesterone secreted into the medium was determined by radioimmunoassay. ●, Response to human LH (mean values ± S.D. of quadruplicate determinations); ○, response to the β-subunit (mean values ± S.D. of duplicate determinations).

Table 1. *Inhibition of ovarian response to human or ovine lutropin (LH) by human chorionic gonadotropin β-subunit*

For experimental details see the text. The primed ovarian system was from a rat treated with pregnant-mare-serum gonadotropin. Inhibition = 100 × (response to LH − response to LH + *β*-subunit)/response to LH. Values are means of duplicate determinations.

Expt. no.	Hormone (µg/ml)	Progesterone (ng/2h per mg of tissue)	Inhibition (%)
Immature ovarian system			
1	—	0.44	
	Human LH (2.5)	11.95	
	Human LH (2.5) β-Subunit (5.0)	5.63	53
2	—	0.22	
	Human LH (2.5)	17.88	
	Human LH (2.5) β-Subunit (5.0)	8.70	51
3	—	0.87	
	Ovine LH (2.0)	6.06	
	Ovine LH (2.0) β-Subunit (5.0)	2.87	53
Primed ovarian system			
1	—	2.56	
	Human LH (2.5)	27.05	
	Human LH (2.5) β-Subunit (5.0)	18.30	32
2	—	2.35	
	Human LH (2.5)	30.83	
	Human LH (2.5) β-Subunit (5.0)	20.58	33
3	—	2.17	
	Human LH (2.5)	22.45	
	Human LH (2.5) β-Subunit (5.0)	13.33	41

creasing concentrations of ovine lutropin, *β*-subunit concentration being kept constant at 5.0 µg/ml. At the end of incubation, progesterone secreted into the medium was determined. The tissue was washed four times with 2 ml of chilled 0.05 M-phosphate buffer in saline containing 0.05 M-EDTA, pH 7.4, homogenized in the same buffer and processed for tissue-bound lutropin by using the method standardized by Muralidhar & Moudgal (1976*a*). Ovine lutropin was measured by using a homologous antiserum raised in our laboratory. This antiserum had very little cross-reactivity (< 5.0%) with the *β*-subunit at the dilution used in the assay (1/45000 initial dilution); ovine lutropin (given by Dr. M. R. Sairam) was used as the standard. At a concentration of 0.1 µg of ovine lutropin/ml of medium, there was a 5-fold increase in progesterone production (Fig. 3). However, at this dose the actual amount of the hormone bound was hardly detectable. At all higher doses tried, there was a dose-dependent increment in binding, but the

degree of increment in the response was not proportional to binding. Moreover, the response reached saturation with 1.0 µg of ovine lutropin/ml, whereas the binding did not reach saturation even with 10 µg of ovine lutropin/ml. When *β*-subunit was added there was a marked inhibition of binding (50%) only at 10 µg of ovine lutropin/ml.

Specificity of the inhibitory effect

To ascertain that the effect observed was specific to the *β*-subunit, the ability of *α*-subunit to bring about inhibition of response, if any, was tested. Ovaries pooled from pregnant-mare-serum-gonadotropin-primed rats were incubated first with 5.0 µg of *α*-subunit/ml for 30 min; ovine lutropin was then added and incubation continued for a further 2 h period. No inhibition was observed (Table 3). Since the *α*-subunit was itself biologically quite active, it was treated with rabbit serum antibody to ovine lutropin *β*-subunit (*γ*-globulin fraction) rendered insoluble by coupling to Affigel-10 (Bio-Rad Laboratories, U.S.A.). Such a treatment removed gross contamination of the *α*-subunit with undissociated human chorionic gonadotropin, resulting in a significant reduction in its biological activity (Table 3). This preparation, once again, was not able to inhibit the response to lutropin (Table 3).

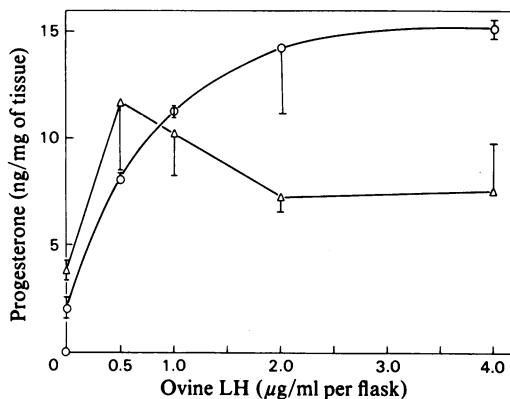Further, in order to rule out the possibility that the

Fig. 2. *Dose–response curve for ovine lutropin (LH) in the presence (△) and the absence (○) of human chorionic gonadotropin β-subunit*

Minces of ovarian tissue obtained from pregnant-mare-serum-gonadotropin-primed rats were incubated in 1 ml of incubation medium, each flask containing 15–16 mg of tissue, with or without 5.0 µg of the *β*-subunit for 30 min at 37°C. Ovine LH was then added and incubation was continued for 2 h. Response was monitored as described in Fig. 1. Values are means ± S.D. from quadruplicate determinations.

Table 2. *Effect of increasing concentrations of human chorionic gonadotropin β-subunit on the response to human or ovine lutropin (LH)*

Minces of ovarian tissue obtained from pregnant-mare-serum-gonadotropin-primed rats were first incubated with increasing concentrations of α- and β-subunit (1–10 μg/ml). Human or ovine LH was then added and incubation was continued for 2 h. Progesterone secreted into the medium was determined by radioimmunoassay. In the upper section of the Table mean results ± s.D. are shown (significance of difference from response to human LH alone:* not significant; ** $P<0.05$; *** $P<0.01$). In the lower section of the Table mean values of results from duplicate flasks are shown.

Expt. 1 (n = 4)		Expt. 2 (n = 3)	
Hormone (μg/ml)	Progesterone (ng/mg of tissue)	Hormone (μg/ml)	Progesterone (ng/mg of tissue)
Human LH (2.0) (Papkoff)	28.5 ± 2.37	Human LH (1.0) (Sairam)	11.67 ± 1.45
Human LH (2.0)+ β-subunit (1.0)	23.25 ± 4.35*	Human LH (1.0)+ β-subunit (2.5)	8.3 ± 0.7**
Human LH (2.0)+ β-subunit (2.5)	23.7 ± 5.3*		
Human LH (2.0)+ β-subunit (5.0)	19.3 ± 5.30**	Human LH (1.0)+ β-subunit (5.0)	7.2 ± 0.65***
Human LH (2.0)+ β-subunit (10)	11.0 ± 2.1***		

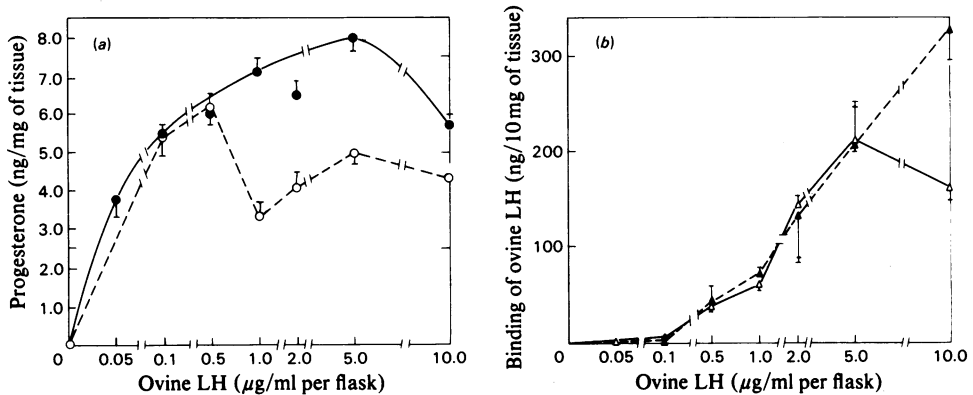|	Progesterone (ng/mg of tissue)	
Hormone (μg/ml)	Expt. 1	Expt. 2
Ovine LH (1.0)	13.7	15.00
Ovine LH (1.0)+β-subunit (5.0)	11.5	10.00
Ovine LH (1.0)+β-subunit (10)	8.3	7.5

Fig. 3. *Correlation between (a) net response to and (b) binding of ovine lutropin (LH) in the presence and the absence of human chorionic gonadotropin β-subunit*

Minces of ovarian tissue obtained from pregnant-mare-serum-gonadotropin-primed rats were incubated with ovine LH in the presence and absence of the β-subunit in the same way as described in Fig. 2, and the response was also monitored in the same way. (a) Net responses (●, ○) were calculated after deducting the response observed when no ovine LH was added or the response observed when the β-subunit alone was added to the incubation flasks: ●, ovine LH alone; ○, ovine LH+β-subunit (5.0 μg/ml). (b) To determine ovine LH bound, ovarian tissue was washed four times with cold 0.05 M-phosphate/0.05 M-EDTA in saline buffer (pH 7.4) and tissue-bound ovine LH was determined by radioimmunoassay carried out at 37°C as described by Muralidhar & Moudgal (1976a). ▲, Ovine LH binding alone; △, ovine LH binding in the presence of the β-subunit (5.0 μg/ml). Values are means ± s.D. of quadruplicate determinations.

Table 3. *Effect of human chorionic gonadotropin α-subunit on the response to ovine lutropin (LH)*

Minces of ovarian tissue obtained from pregnant-mare-serum-gonadotropin-primed rats were incubated with or without α-subunit (CR-119). One set of flasks (third line of Table) received the α-subunit (CR-119) purified further by treatment with rabbit antibody (γ-globulin fraction) to ovine lutropin β-subunit coupled with Affigel-10. Ovine LH was then added and the response was determined as described in Fig. 1. Mean values ± S.D. of triplicate determinations are shown. n.s., No statistically significant difference from the result with ovine LH alone (top line).

Hormone (μg/ml)	Progesterone (ng/2h per mg of tissue)
Ovine LH (1.0)	12.19 ± 1.86
α-Subunit (5.0)	6.87 ± 0.25
α-Subunit (further purified) (5.0)	3.23 ± 0.24
Ovine LH (1.0) + α-subunit (5.0)	11.03 ± 1.11 (n.s.)
Ovine LH (1.0) + α-subunit* (5.0)	13.57 ± 1.10 (n.s.)

* Treated with anti-(ovine lutropin β-subunit) serum globulins coupled with Affigel-10.

Table 4. *Effect of removal of excess of human chorionic gonadotropin β-subunit from the medium on the subsequent response to ovine lutropin (LH)*

Minces of ovarian tissue obtained from pregnant-mare-serum-gonadotropin-primed rats were first incubated in 1 ml of incubation medium containing β-subunit (5.0 μg) for 30 min. The medium was discarded, the tissue was blotted on a wet filter paper to remove excess of medium and suspended in fresh medium containing ovine LH (1.0 μg/ml), and incubation was continued for 2 h. The response was determined as described in Fig. 1. Results are shown as means ± S.D. *$P \approx 0.05$.

Hormone (μg/ml)	Progesterone (ng/mg of tissue)
Nil	0.52 ± 0.2
Ovine LH (1.0)	12.87 ± 3.27*
Ovine LH (1.0) + β-subunit (5.0)	6.97 ± 1.47*

inhibition brought about by the human β-subunit is not due to non-specific interaction between the free β-subunit and the hormone, the tissue was first suspended in medium containing β-subunit and incubated at 37°C for 30 min, after which the medium was discarded. The tissue was then carefully blotted on a wet filter paper to remove any excess of medium and then transferred to fresh medium containing only
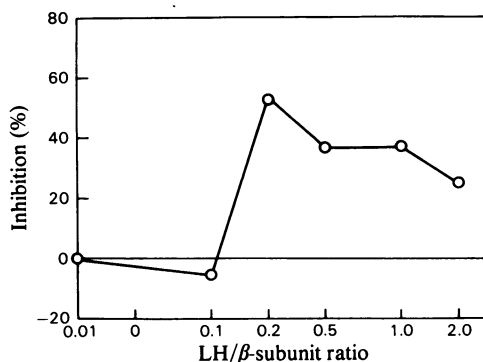

Fig. 4. *Percentage inhibition of response as a function of the lutropin/human chorionic gonadotropin β-subunit ratio* The inhibition of response observed in Fig. 3 was plotted as a function of the ratio. Details of calculation of the percentage inhibition of response are given in Table 1.

ovine lutropin. The incubation was continued for a further period of 2 h. Although free β-subunit was not present in the final incubation medium, significant inhibition in response ($\sim$46%) could still be observed (Table 4).

Discussion

The results presented above show that the β-subunit of human chorionic gonadotropin, by virtue of its ability to bind to lutropin receptors, inhibits the steroidogenic response to the whole hormone. The inhibition of response observed is a dose-dependent phenomenon, the maximal inhibition being observed with 10 μg of the β-subunit/ml of medium. The β-subunit in itself, at all doses tested, had minimal steroidogenic activity (equivalent to less than 10 ng of human lutropin). The inhibitory activity was observed in both the rat ovarian systems used and the β-subunit was capable of inhibiting response to both human and ovine lutropin. This corroborates our earlier observations with ovine β-subunit with regard to its ability to inhibit binding and response (cyclic AMP production) to the native hormone and permits us to conclude that the β-subunits of human chorionic gonadotropin and lutropin have a minimal topography necessary for it to be recognized by lutropin receptors.

The specificity of the effect brought about by the subunit has been verified in several ways. The fact that removal of unbound (excess) β-subunit from the medium did not negate the observed inhibition would suggest that the effect of the β-subunit is not due to any non-specific interaction between the

subunit and the whole hormone, but is, in fact, due to the occupancy of the relevant receptors by the β-subunit. Since β-subunit is known to form aggregates under some conditions, gel-filtration profiles of ^{125}I-labelled hormone and the β-subunit individually and as a mixture (mixed in the same proportions as used in the inhibition experiments), were determined on Sephadex G-100. The results showed that, within the period of incubation (2h), there was no detectable interaction between the whole hormone and the subunit, which could be seen by a shift in the elution profile (data not shown). The specificity of the effect of the β-subunit was further confirmed by the observation that highly purified human chorionic gonadotropin α-subunit, when tested under identical conditions, did not show any inhibitory activity.

Earlier work from our laboratory clearly showed that the amount of β-subunit that binds to lutropin receptors is much lower than that for an equivalent concentration of the native hormone (Muralidhar & Moudgal, 1976a,b,c). Thus, at the concentration of the β-subunit used in these experiments, a very small population of the receptors must be occupied by the β-subunit. Assuming that response is directly proportional to the occupancy of receptors, to obtain minimal response it is possible that a small population of receptors need only be occupied by the hormone. This can presumably be achieved even in the presence of the β-subunit, and consequently at such concentrations inhibition of response may not be observed. However, when the response expected is very high or maximal, it is to be assumed that receptor occupancy should also be proportionally high or optimum. The occupancy of a proportion of receptors by the β-subunit in such cases may interfere with whole lutropin occupying the required number of receptors, resulting in an observed inhibition of response. At low hormone concentrations (1–5 μg of lutropin/ml) it has not been possible to monitor the inhibition of binding of ovine lutropin (of a low order of magnitude), perhaps owing to lack of discriminative ability of the radioimmunoassay used at this concentration. Despite this, because the response is an amplified effect of binding, an inhibition in response is observed. At a high concentration of lutropin (10 μg/ml) inhibition of both binding and response can be observed, thus supporting our suggestion above.

Another evidence in favour of our suggestion is the observation that, for a given concentration of lutropin, the degree of inhibition obtained is dependent upon the number of receptors available (Table 1). The reason for observing a higher degree of inhibition (55 %) in the immature ovarian system is perhaps a reflection of less receptors being present in this

system as compared with those available in the pregnant-mare-serum-gonadotropin-primed ovarian system. Further, it appears from these studies that a critical ratio of lutropin/β-subunit is a prerequisite for obtaining significant inhibition. As can be seen from Fig. 4, the inhibition becomes evident when this ratio is 0.2 or higher.

The observation that excess of hormone is not able to over-ride the inhibitory effect of the β-subunit totally can be taken as supporting evidence for the strength of the subunit binding to the receptor, as well as the specificity of binding. All the same, the present results are rather interesting if one accepts the current thinking that all the receptors are equivalent in terms of binding and response. It is possible that each response to a hormone is associated with a specific class of receptors(?) with its own affinity, or more likely with a varying degree of occupancy. If this is so then the observed effect refers to blocking of a special class of receptors involved in the amplification of steroidogenic response by the β-subunit. It is, however, necessary to analyse the effect of β-subunit binding on a broad spectrum of responses which can be stimulated in vitro, before a definite conclusion can be drawn as to whether there are different receptors for different responses.

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