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β subunits of human choriogonadotropin and ovine lutropin are biologically active

(Leydig cell assay/testosterone/affinity chromatography/HPLC)

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ABSTRACT **Highly purified β subunits of human choriogonadotropin and ovine lutropin were found to possess measurable steroidogenic activity in the *in vitro* rat Leydig cell assay. In addition, β subunits of these two gonadotropins were able to inhibit the biological activity of human choriogonadotropin in the rat Leydig cell assay.**

Ever since the subunit nature of ovine lutropin (oLH) was discovered (1, 2), conflicting reports have appeared as to the intrinsic biologic activity of the β subunit. Earlier workers have shown that oLH-β exhibits lutropic activity in *in vivo* (3, 4) and *in vitro* (5, 6) systems. Such activity has primarily been ascribed to contamination of the subunit with the intact hormone (7, 8). In light of recent reports that β subunits of both oLH and human choriogonadotropin (hCG) do exhibit activity inhibitory to hCG (9, 10), it was considered worthwhile and important to investigate this problem using highly purified β subunit preparations of hCG and oLH freed of possible contamination with intact hormones. Results of these studies are herein reported.

MATERIALS AND METHODS

Hormones. hCG and its β subunit preparation were obtained from Robert Canfield through the courtesy of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. oLH and its subunits were prepared as described (11).

HPLC. HPLC was performed according to standard procedures (12, 13) using a C_8 (330 Å) column (4.5 × 250 mm). Elution of the injected protein was achieved by using a linear gradient of *n*-propanol (10–40%). The pooled fractions were lyophilized and the recovered material in some instances was further purified by chromatography on Sephadex G-100 (1.4 × 60 cm) in 0.01 M NH_4HCO_3 at pH 8.4.

Affinity Chromatography. Two batches of antibody to hCG-α, one completely free of antibodies to hCG-β but still capable of binding intact hCG (preparation A) and the other showing crossreactivity with oLH (preparation B) were coupled to Affi-Gel-10 (Bio-Rad) as described (14). hCG-β and oLH-β preparations were treated with the hCG-α antibody-Affi-Gel preparations A and B, respectively, to remove any undissociated hormone that may have been present. The antibody-Affi-Gel preparations were packed in the form of short columns (1 × 2 cm) in 5-ml disposable plastic syringes. After the subunit had been allowed to sink into the gel, the column was locked and stored overnight at 4°C before elution with phosphate-buffered saline (P_i/NaCl). This column could be regenerated by treatment with P_i/NaCl containing 3 M KI as described (15).

Leydig Cell Assays. Isolation of Leydig cells from rat testes (two rats; 80 and 100 days old) and the Leydig cell assays were essentially as described (16) with minor modifications (17). At the end of the 2-hr incubation period, the cells with the medium were frozen until they were used for determination of testosterone by radioimmunoassay (17).

RESULTS

Characterization of hCG and oLH β Subunits. Two milligrams each of hCG (CR123), hCG-α, and hCG-β were subjected to HPLC, and the eluted material was pooled and lyophilized. Recovery of the peak fractions ranged from 60% to 75%. It is evident from the HPLC profiles of hCG and its subunits (Fig. 1) that the intact molecule can be clearly separated from its subunits by using the system described here. The oLH subunits were also subjected to HPLC; although their elution profiles did not show clear separation of the subunits from one another, considerable purification was achieved by restricting to narrow cuts and rechromatographing the eluted material by HPLC and on a Sephadex G-100 column.

The capacity of the hCG-α antibody (preparation A) immunoaffinity column for removing intact hCG, a possible contaminant of hCG-β, was assessed by allowing 10 μg of hCG in 0.1 ml of P_i/NaCl to sink into the column gradually, storing the column overnight at 4°C, and washing with 10 ml of P_i/NaCl. The wash was pooled and assayed for hCG in an appropriate radioimmunoassay. One passage through the column resulted in 95% of the loaded hCG being adsorbed by the immunoaffinity gel. On the basis of the Leydig cell assay, the 750 μg of hCG-β passed through this column could have been contaminated with no more than 4.5 μg of hCG, and consequently one passage through the column was considered adequate to remove all of the hCG contamination. A second passage through the affinity column was performed to ensure complete removal of the contamination.

The oLH-β was treated with a hCG-α antibody (preparation B) coupled to Affi-Gel-10 in a like manner to remove any intact oLH that could be present in the preparation. The capacity of this antibody to bind oLH was approximately 50% of that of the first antibody Affi-Gel column. oLH-β consequently had to be passed through the column twice.

Steroidogenic Activity on the Subunits. Despite further purification by HPLC and treatment with an antibody capable of

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Abbreviations: LH, lutropin; oLH, ovine LH; hCG, human choriogonadotropin; LH-β, β subunit of LH; hCG-β, β subunit of hCG; hCG-α, α subunit of hCG; P_i/NaCl, 0.01 M phosphate-buffered saline, pH 7.4.

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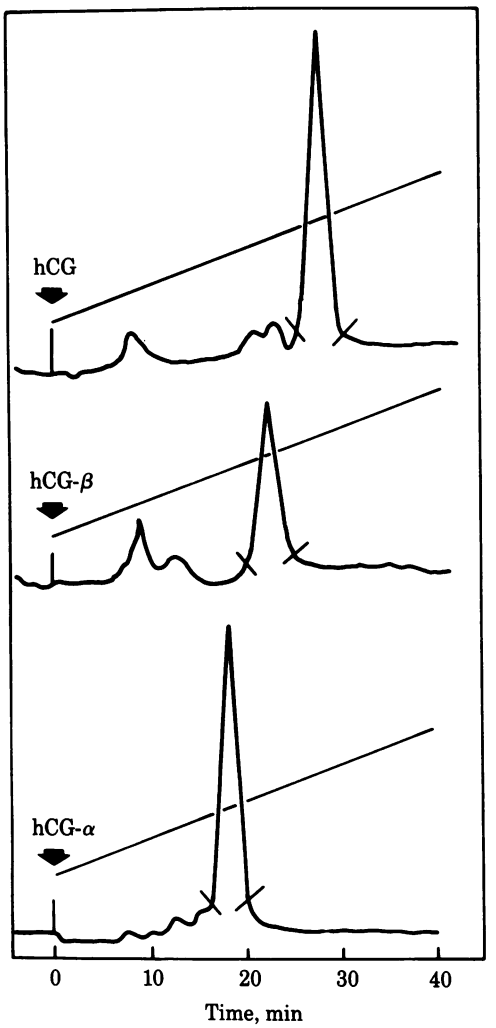

FIG. 1. Elution profile of hCG (100 μg) and its subunits (100 μg) on HPLC using a C_8 (300 Å) column (4.5 × 250 mm). Elution was achieved with a linear 10–40% gradient of *n*-propanol. Rechromatography of the eluted material by HPLC gave a homogeneous peak.

removing the intact hormone, the β subunits of hCG and oLH gave a dose-dependent response (Figs. 2 and 3). The immunoaffinity chromatography-purified oLH-β-C was as active as oLH-β-A (unfractionated) at 1,000 ng but its activity at 100 ng was 30% of that of an equivalent dose of oLH-β-A. The hCG-β-C preparation also was tested for its steroidogenic activity in a rat granulosa cell system and found to be active [mean ± SD ng of progesterone produced per ml per 2 hr: control, 7.34 ± 0.76; hCG-β-C (400 ng), 29.36 ± 6.46].

Ability of Subunits to Inhibit Response to Intact Hormone. hCG-β and oLH-β, in doses that did not in themselves exhibit appreciable steroidogenic activity in Leydig cells, suppressed the response to intact hCG (Fig. 4). In the case of hCG, the maximal suppression in response was obtained when the dose of hCG was saturating (0.5–1.0 ng) and the inhibition ranged from 33% to 60% depending upon the subunit; at the lower concentration of hCG (0.1–0.25 ng) the inhibition obtained was minimal.

DISCUSSION

Attempts were made in the present study to establish that β subunits of oLH or hCG possess intrinsic biological activity. In order to ascertain that the β subunit preparations used were free of contamination, HPLC followed by immunoaffinity procedure was used to remove minute contamination, if any, with the native hormone. After passing through these steps of purification, both hCG-β and oLH-β still exhibited steroidogenic activity (Figs. 2 and 3). These observations suggest that the activity exhibited by these subunits, although low (0.01–0.06% in the case of hCG-β), is intrinsic to the molecule. Perhaps it is for this reason that earlier workers assaying the different β subunits at the 10- to 50-ng level failed to observe any steroidogenic activity. However, Ramakrishnan *et al.* (18) have shown that an immunoaffinity-purified hCG-β preparation does exhibit steroidogenic activity when assayed at a high level (400 times the dose of hCG) in the mouse Leydig cell assay.

The observation regarding the ability of the β subunit of hCG or oLH to inhibit gonadal cell responsiveness to the intact hormone essentially confirms and extends our earlier findings (9, 10). The inhibitory effect has been observed at the β subunit

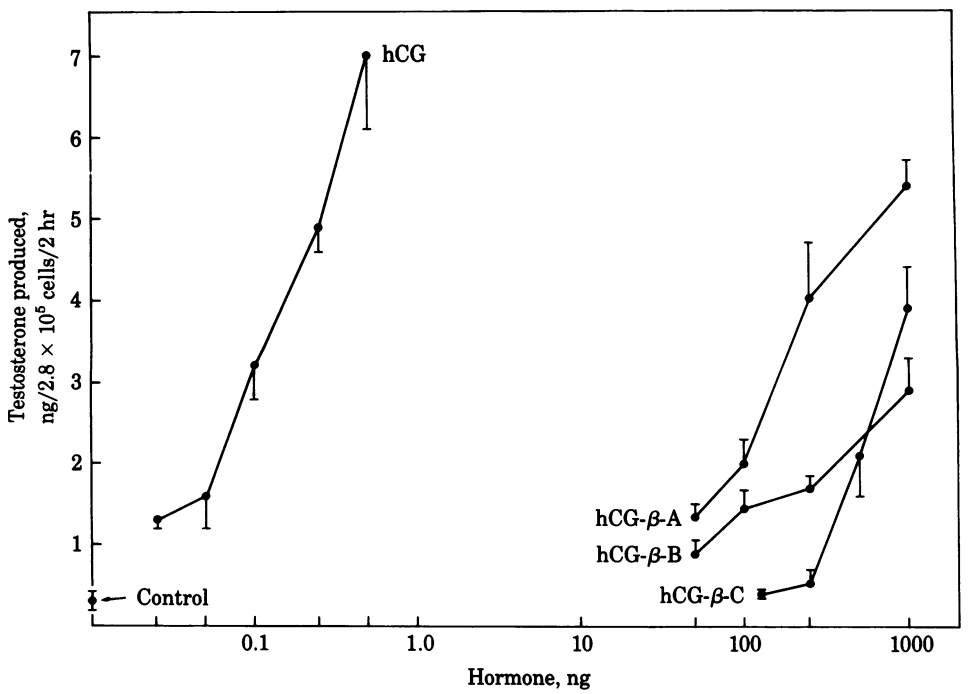

FIG. 2. Ability of hCG-β to stimulate testosterone production in the rat Leydig cell assay. hCG and hCG-β-A refer to the original preparations (CR123). hCG-β-B and hCG-β-C refer to HPLC-fractionated and immunoaffinity preparations, respectively. Results are shown as mean with SD.

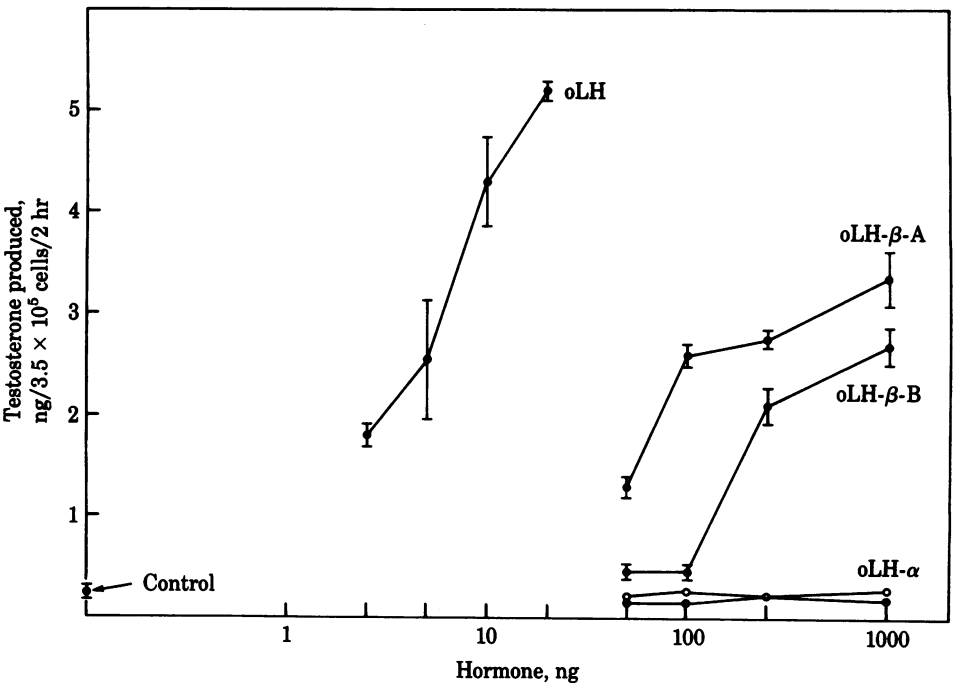

FIG. 3. Ability of oLH-β to stimulate testosterone production in rat Leydig cells. oLH-β-A, starting material; oLH-β-B and oLH-α, HPLC-purified preparations. Note that the α subunit was not active in this system at the dose tried. Results are shown as mean ± SD.

concentration range of 10–100 ng, a level at which the β subunit has minimal stimulatory activity. Inhibition studies at higher concentrations of the β subunit could not be carried out because the subunit itself exhibited steroidogenic activity at these levels.

It has been shown that, when highly purified hCG-β is injected into hypophysectomized-estrogenized female rats, the ovarian follicle specifically picks up hCG-β. The localization of the β subunit was achieved by an immunohistochemical technique using specific hCG-β and hCG-α antisera (unpublished data).

These findings are in keeping with the overall premise that a part of the whole molecule can be expected to inhibit, at least partially, the activity of the parent molecule. There are several such examples in the case of polypeptide hormones. A corti-

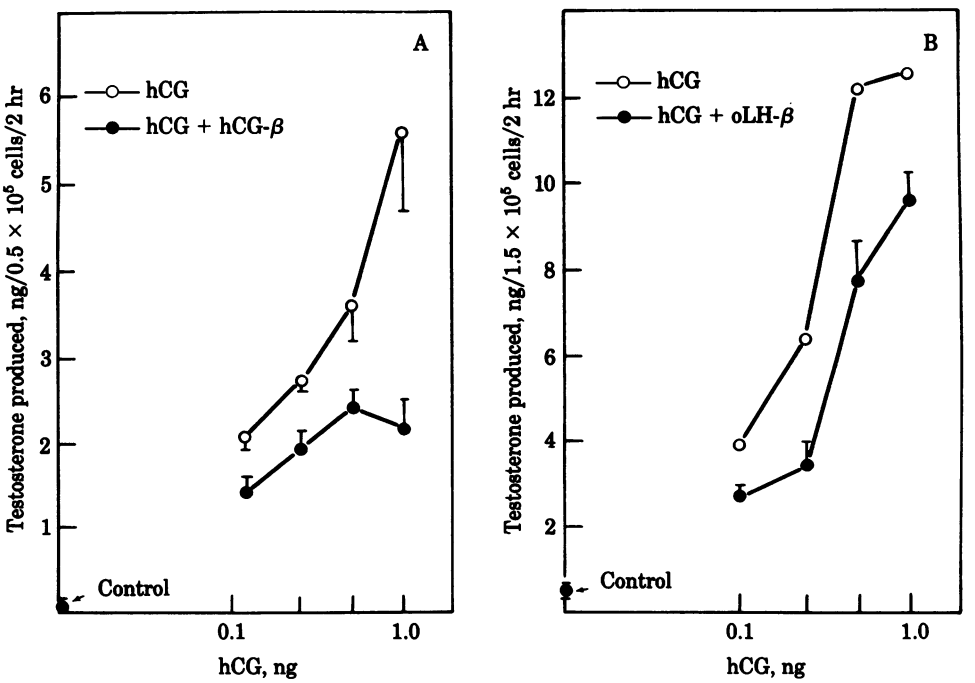

FIG. 4. Ability of β subunits of hCG and oLH to inhibit steroidogenic response of Leydig cells to hCG. The cells were preincubated for 30 min at 37°C with 100 ng of hCG-β (purified by HPLC) and 25 ng of oLH-β (purified by immunoaffinity). The β subunits at these levels produced the following response (mean ± SD): controls, 0.094 ± 0.67 ng of testosterone per 2 hr; 100 ng of hCG-β, 0.520 ± 0.17; 25 ng of oLH-β, 0.664 ± 0.09. Data points are means with SD.

cotropin-inhibiting peptide has been isolated from human pituitary extracts and its structure was shown to be α_h-ACTH-(7–38) (19). A segment of β-endorphin [β-endorphin-(6–31)] is an inhibitor of the analgesic activity of the parent peptide (20).

Partially deglycosylated oLH-β recently has been shown to antagonize the action of the intact hormone as assessed by steroidogenesis in the dispersed rat Leydig cells *in vitro* (21). It may be worthwhile to determine if such chemical modification increases the inhibitory activity of the β subunit of hCG against the intact hormone.

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