Complete dissociation of gonadotropin receptor binding and signal transduction in mouse Leydig tumour cells

Obligatory role of glycosylation in hormone action

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Utilizing a clonal cell line of mouse testicular Leydig cells (MA-10 cells) the complete steroidogenic and other hormonal properties of chemically deglycosylated ovine lutropin (DG-LH) and human chorionic gonadotropin (DG-hCG) were evaluated. In these cells, with the LH receptor–steroidogenic mechanism tightly coupled and in which there are few, if any, spare receptors, both DG-LH and DG-hCG failed to elicit progesterone production, unlike fully glycosylated native LH and hCG. The receptor-binding activity of DG-LH and DG-hCG was 2–3 times that of LH and hCG in competition experiments with radiolabelled hormones. The typical phenomenon of rounding of MA-10 cells induced by LH and hCG was absent when cells were incubated with DG-LH or DG-hCG. This could be directly attributable to their failure to produce cyclic AMP as second messenger. DG-LH and DG-hCG inhibited cell shape changes and steroidogenesis caused by LH and hCG. The deglycosylated hormones were potent antagonists of the action of glycosylated hormones. Delaying DG-hCG (antagonist) addition for up to 1 h after initiation of hCG action was also very effective in preventing further activation of steroidogenesis. Similar effects were produced by addition of affinity-purified anti-hCG antibodies. In affinity cross-linking experiments, both hCG and DG-hCG bound to the same 90 kDa receptor. Studies with MA-10 cells thus provide unequivocal evidence that the presence of antennary sugars in LH and hCG (and perhaps in other similar hormones such as follicle-stimulating hormone and thyroid-stimulating hormone), is essential for signal transduction. Differences observed in the literature in other cellular systems may be attributed to differences in hormone–receptor–effector coupling.

INTRODUCTION

Pituitary gonadotropins [follicle-stimulating hormone (FSH) and luteinizing hormone (LH)] and thyrotropin (thyroid-stimulating hormone, TSH) as well as their placental counterparts (such as human chorionic gonadotropin) (hCG) are unique among large protein hormones. Of the two non-covalently linked α - and β -subunits present in the dimeric structure, the α -subunit is common to these hormones in the pituitary and placenta. In addition, the subunits contain a high content of oligosaccharide units, of both the *N*-linked as well as *O*-linked variety, depending upon the hormone and tissue [1–4]. The individual subunits with their carbohydrate units fully intact are virtually inactive in most biological tests of hormone action, including receptor binding, and hence the proper recombination of the α - and β -subunits is necessary for efficient interaction with high-affinity receptor sites on the target cell membrane [1–4].

As glycosylation of proteins is ubiquitous in eukaryotic systems, it is believed that the oligosaccharide moieties of glycoproteins may play a significant role in widespread biological phenomena such as circulatory half-life, cell–cell recognition, stabilization of protein structure and intracellular trafficking [5,6]. Anhydrous HF [2] and

similar reagents [7] have been applied to study the functional role of oligosaccharides in glycoprotein hormones [8]. Several exoglycosidases have also been used for shortening the oligosaccharides of hCG [9,10] or its subunits [11]. Such enzymes cannot be used with pituitary hormones because of the presence of sulphated hexosamines [1,12], but chemical deglycosylation is highly effective [12]. These partial deglycosylation studies have revealed that the carbohydrate chains in their fully extended form impart the following properties: (1) they are not essential for intersubunit recognition and thus formation of the dimeric structure, (2) they are not required for receptor recognition, (3) their removal does not alter immunoreactivity as tested by antibodies to native hormones, but (4) removal causes definite detrimental effects with regard to catalysing intracellular events such as adenylate cyclase activation or steroidogenesis, or other cellular responses [8]. Further studies involving differential deglycosylation of the subunits also revealed that glycosylation of the α -subunit is critical for hormone signal transduction [13]. This concept has been extended by oligonucleotide-directed mutagenesis studies to show that the first site of glycosylation from the *N*-terminus of the α -subunit is more important for hormone activity [14].

Abbreviations used: DG, deglycosylated; (h)CG, (human) chorionic gonadotropin; (o)LH, (ovine) luteinizing hormone (lutropin); FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone (thyrotropin).

Most studies recognize that although second messenger generation (cyclic AMP) is severely compromised following deglycosylation, variable responses in steroidogenic activity of deglycosylated (DG) LH and hCG are noted in different cellular systems from species ranging from rats to humans [2,7–22]. These may be divided into two broad groups. (1) A low potency type in which the deglycosylated hormone (e.g. DG-hCG) has low activity but produces the same maximum as that induced by the native hCG [16,20,22]. (2) A type in which the DG-hormone (e.g. DG-LH, DG-hCG) is reportedly not only less active ($< 1\text{--}5\%$) but also less efficacious [2,10–12,14,15,19,21], as maximum steroidogenesis cannot be achieved. In contrast with these studies, one report [23] employing endoglycosidase F-treated ovine and human LH preparations found little decrease in their steroidogenic potency and concluded that the carbohydrates of the hormone were not important for function. In order to understand the basis of some of these differences, a detailed study of the action of DG-LH and DG-hCG and their native hormones has been performed in an identical setting using mouse Leydig tumour cells. Chemically deglycosylated oLH and hCG are of particular interest in this investigation, because their preparations have been subjected to extensive biochemical characterization [2,7,12,15,16,18,19,24]. Using a clonal cell line in which the receptor-effector coupling is in a state of apparent identity in all cultured cells, a situation which is difficult to achieve in normal gonadal tissue, it can be shown that the lack of antennary carbohydrates in both hormones leads to a complete dissociation of receptor binding and steroidogenic response.

MATERIALS AND METHODS

Hormones and deglycosylated preparations

Highly purified oLH and DG-oLH [24] as well as hCG and DG-hCG [15] were prepared in this laboratory by treatment of the hormone with anhydrous HF according to published procedures [15]. Each of these DG preparations was chromatographed on concanavalin-A-Sepharose to exclude the presence of fully glycosylated native hormone. Under our conditions of chromatography, DG-hormones emerge in the unabsorbed fraction, which is again purified on Sephadex G-100. The α - and β -subunits of oLH were prepared by reverse-phase h.p.l.c. [25]. All preparations were stored at 4°C and weighed precisely on a Cahn electrobalance as and when needed.

Mouse interstitial cells

These cells, which are the targets of LH/hCG action, were prepared by mechanical disruption of decapsulated testes from 3-month-old mice (Charles River, St. Constant, Quebec, Canada) [26] and incubated in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, U.S.A.). The cells were incubated with the hormone or variants for 2 h at 34°C , after which the contents were boiled and the supernatant utilized for measurement of cyclic AMP by the protein binding assay method [27], or of testosterone by a specific radioimmunoassay [15]. The cyclic AMP binding protein was a bovine adrenal preparation donated by Dr. A. Goff (St. Hyacinthe, Quebec, Canada). Using this preparation, the sensitivity of the assay was 0.1 pmol/tube .

Mouse Leydig tumour cells

The MA-10 clone of mouse Leydig tumour cells was provided by Dr. M. Ascoli (Population Council, New York, NY, U.S.A.). The frozen cells were propagated and maintained according to his procedures with some modifications [28]. MA-10 cells were able to grow well for 3 days in Optimedium (Gibco) supplemented with 10% horse serum. The only time these cells were exposed to any enzyme was a brief treatment with trypsin to dislodge them from the T-75 flasks for plating on to 24-well plates (Nunc). On the day of the experiment, spent medium was removed and the cells were washed twice with serum-free medium. Cells were then incubated at 37°C in the presence of stated additives in a total volume of $500\text{ }\mu\text{l}$ of serum-free medium for specified intervals. Progesterone secreted into the medium was estimated by radioimmunoassay [15]. The antibody used for this purpose was kindly donated by Dr. B. E. P. Murphy, Montreal General Hospital, Montreal, Canada. Its cross-reactivity with 20α -hydroxyprogesterone was $< 0.05\%$.

In some experiments, MA-10 cells were also incubated for 24 h, after which they were examined under the phase-contrast microscope and photographed. In studies designed to evaluate the antagonistic behaviour of DG-hormones, different concentrations were added at the same time along with a challenging dose of LH or hCG. After 4 h, the progesterone formed was estimated. Other variations from this protocol are given in the legends to appropriate Figures.

Hormone binding to MA-10 cells

hCG/DG-hCG binding to mouse Leydig cells was evaluated by using ^{125}I -labelled hormones. For this purpose, hCG iodinated by the lactoperoxidase method [15] and purified on Sephadex G-50 (Pharmacia, Dorval, Quebec, Canada) or on a Bio-Rad P6DG column (Richmond, CA, U.S.A.) was used. Application of the same method of fractionation to DG-hCG was unsuitable because of higher non-specific binding to several test receptors such as homogenates of rat testes or pig granulosa cells. This was overcome by fractionation on SP-Sephadex C50 (Pharmacia) in 0.033 M -acetate buffer, pH 5.4. The adsorbed iodinated hormone was eluted by $0.1\text{ M-Na}_2\text{HPO}_4/0.3\text{ M-NaCl}$ in the presence of 1 mg of bovine serum albumin/ml (Sigma, St. Louis, MO, U.S.A.). The specific activities of the above preparations ranged between 40 and $65\text{ }\mu\text{Ci}/\mu\text{g}$, and they showed a specific binding of $70\text{--}80\%$. The labelled hormones were stored frozen at -70°C and were usually used within 2–3 weeks of preparation.

In direct competition experiments, inhibition of binding of labelled hCG to MA-10 cells by unlabelled hCG, DG-hCG, LH or DG-LH was compared. In other experiments, the site(s) of binding of labelled hCG and DG-hCG to receptor components on MA-10 cells was compared by performing cross-linking with disuccinimidyl suberate (Pierce, Rockford, IL, U.S.A.) [29]. The cells containing the cross-linked hormone was washed and treated with 4% SDS to solubilize membrane components. The extract supernatant was subjected to SDS/polyacrylamide-gel electrophoresis (PAGE) analysis [15] in $5\text{--}15\%$ gradient gels. The gels were dried and exposed to X-ray film (Kodak XAR-5) with an intensifying screen at -70°C for about 3 weeks. Appro-

appropriate controls were included in each experiment and these are shown in the relevant Figure legends.

RESULTS

Cellular activation in normal mouse Leydig cells and in tumour cells

In homogenates of normal mouse testes or MA-10 tumour cells grown as solid tumours in mice, DG-hCG was 2–3-times more potent than hCG in competing with ^{125}I -hCG for binding sites. Similar results were obtained with MA-10 cells plated in culture during a 3 h competition assay (results not shown). These data corroborate previous results obtained using a number of other LH receptor systems [8].

In a test system wherein normal mouse Leydig cells were obtained by gentle mechanical dispersion alone and used without any further purification, the effects of hCG and DG-hCG on both second messenger (cyclic AMP) generation and testosterone secretion were clearly different (Fig. 1). For hCG, induction of steroidogenesis occurred at concentrations which caused little cyclic AMP accumulation in the medium. DG-hCG on the other hand did not increase cyclic AMP formation at any of the concentrations examined. The effects of DG-hCG on testosterone were different from those of hCG in two respects. First, the potency of DG-hCG in terms of hCG was less than 2% as calculated from the linear portion of the dose-response curve and, secondly, steroidogenesis did not attain the same maximum with DG-hCG as with hCG in all experiments and this difference was significant ($P < 0.02$).

In order to compare the steroidogenic effects in detail,

Fig. 1. Biological effects of hCG and DG-hCG in normal mouse Leydig cells

Cells prepared by gentle mechanical disruption of testes were incubated in Dulbecco's modified Eagle's medium for 2 h at 34 °C in the presence of O_2/CO_2 (19:1) in a total volume of 300 μl . Cyclic AMP in (b) was estimated by the protein binding assay and testosterone in (a) was determined by a radioimmunoassay. Each point is a mean $\pm$ s.d. of triplicate incubations and error bars, where not depicted, are within the symbol.

more elaborate studies were done with mouse Leydig tumour cells. The MA-10 cells in culture responded to the addition of LH/hCG by secreting large amounts of progesterone during a 4 h incubation (Fig. 2a). In all experiments (Fig. 2), different DG-LH/DG-hCG preparations made in the laboratory did not exhibit the same tendency to increase progesterone secretion by MA-10 cells. As there was no concentration-dependent increase of steroid secretion, potency estimates could not be

Fig. 2. Comparison of the steroidogenic effects of hCG, LH, DG-hCG and DG-LH in mouse Leydig tumour (MA-10) cells

(a) Cells grown in 24-well plates were used 3 days after plating. Tumour cells were incubated in serum-free medium (Optimed) with hormones as indicated in a humidified atmosphere for 4 h at 37 °C. Progesterone in the medium was estimated by radioimmunoassay ($n = 3$ per incubation) and similar results were obtained in five other assays. Basal progesterone secretion in the absence of the hormone was 0.36 ng/well. Note that the steroidogenic activity of reverse-phase h.p.l.c.-purified oLH subunits is greater than that of DG-LH. Hormones added: $\blacktriangle$, hCG; $\triangle$, oLH; $\circ$, DG-hCG; $\bullet$, DG-LH; $\blacklozenge$, oLH β -subunit; $\diamond$, oLH α -subunit. (b) Hormone-stimulated progesterone accumulation after 4 and 24 h of stimulation in MA-10 cells. Cells were treated as in (a) with different hormones. At the end of 4 h, 50 μl of medium was removed for steroid assay ($\blacksquare$) and the incubation was continued for 24 h and assayed ($\boxtimes$). The s.d. was 5–8% of the mean value shown for each bar ($n = 3$ per dose). In these cells under active hormonal stimulation, progesterone synthesized during 4 h is subsequently metabolized to the 20 α -hydroxy derivative [28].

Fig. 3. Changes in cell shape of mouse Leydig tumour cells in presence of hormones

MA-10 cells (10^5 /well) were grown as in Fig. 2 and incubated with hCG/LH or DG-hCG/DG-LH in triplicate in serum-free medium for 24 h. The cells were then observed under the light microscope and photographed under identical conditions (magnification $27\times$) (a) Control, (b) 10 ng of oLH, (c) 10 ng of DG-LH, (d) 100 ng of DG-LH, (e) 10 ng of hCG, (f) 100 ng of DG-hCG were added to each well. When 1 mM-dibutyl cyclic AMP was present in the medium in separate wells (results not shown), all cells assumed the rounded shape within 6 h.

calculated. At 100 ng or $1\text{ }\mu\text{g}$ of DG-hormone per well, progesterone secretion was increased only by about 5–10-fold over basal levels as compared with a 400–600-fold increase for LH/hCG at 10 ng/well. In many other experiments (results not shown), DG-LH/DG-hCG addition at $1\text{ }\mu\text{g}$ or $10\text{ }\mu\text{g}$ /well did not produce much of an increase over basal values. In comparison with this, the α - and β -subunits of oLH purified by reverse-phase h.p.l.c. showed a definite (albeit very low) tendency to enhance steroidogenesis (<0.1 and 0.4% respectively relative to native LH) (Fig. 2a). When the incubation was continued for 24 h, progesterone secreted into the medium after LH/hCG addition decreased by about 90–97% due to metabolic degradation [28]. However, in the same experiment, in the presence of DG-LH/DG-hCG, the small amount of progesterone that had been secreted by 4 h persisted at the end of 24 h period (Fig. 2b), indicating that the same set of intracellular events responsible for elevation of progesterone as well as its

subsequent degradation in 24 h were not activated by DG-LH/DG-hCG.

Changes in cell shape

The stimulatory/steroidogenic actions of LH/hCG on mouse Leydig tumour cells are associated with very dramatic changes in the appearance of the cells under the light microscope. Untreated MA-10 cells grew in a sheet-like manner in a polygonal shape with spindle-shaped connections with their neighbours. There were also a few round cells in these wells. Addition of LH or hCG (about $5\times 10^{-10}\text{ M}$) induced a striking rounding effect on the cells in about 6 h, and this process was complete by about 24 h. Virtually all of the cells in the well containing LH/hCG assumed this clear rounded shape (Fig. 3).

In contrast with the effects noted above, DG-LH or DG-hCG at 10 times the concentration used for LH/hCG produced no such changes in cell shape during 24 h (Fig. 3). Such treated cells were barely distinguishable from

Fig. 4. Hormone-antagonistic effect of DG-LH and DG-hCG in MA-10 cells

Cells were plated and incubated with various samples as indicated. MA-10 cells in each well ($n = 3$) were challenged with 1 ng of hCG or oLH in the absence or presence of increasing concentrations of DG-hCG or DG-LH respectively. Both agents (hormone and antagonist) were added to the cells at zero time and the incubation was continued for 4 h. Progesterone accumulated in the medium was analysed by radioimmunoassay.

controls. As all of the changes in cell shape caused by LH/hCG could be fully and rapidly mimicked by the addition of 1 mM-dibutyryl cyclic AMP (results not shown), it is possible to conclude that DG-hormones are unable to induce shape changes because they lack the capacity to activate adenylate cyclase. In the presence of exogenous cyclic nucleotide (1 mM), the cell shape changes were accelerated and completed in about 6 h (results not shown).

Inhibition of progesterone secretion

In the presence of DG-LH or DG-hCG, the progesterone secretion induced by LH or hCG was inhibited in a concentration-dependent manner (Fig. 4). In the experiment shown, a concentration of LH/hCG (1 ng/well) which stimulated progesterone production by about 120-fold over basal was chosen. The nature of the inhibition curve suggested effective inhibition at a 1:1 ratio of glycosylated/deglycosylated hormone. In other experiments (results not shown), it was noted that all of the cell shape changes caused by LH/hCG shown in Fig. 3 could be prevented by the concomitant presence of 10-fold excess of DG-LH/DG-hCG.

The inhibition of LH/hCG-induced progesterone secretion in MA-10 cells was studied further in another

Fig. 5. Efficacy of DG-hCG (a,b) or affinity-purified hCG antibody (c,d) in terminating steroidogenesis in MA-10 cells previously exposed to hCG

Cells were exposed to 1 ng of hCG/well at zero time and DG-hCG (100 ng) or hCG antibody (100 ng) was added at different times as indicated by the arrows. Control wells containing hCG received 50 μ m of medium at these times. Incubation was allowed to proceed for 4 h and the total amount of progesterone accumulated was then measured. The continuous lines in (a) and (c) indicate the time course of steroid accumulation in control wells receiving 1 ng of hCG alone. The broken lines extending to the right represent the level of steroidogenesis permissible under the experimental conditions in the presence of added antagonist (a) or hCG antibody (c). Panels (b) and (d) are plots of the inhibitory response versus the delay in DG-hCG or hCG antibody addition and was calculated as follows:

$$\text{Inhibition (\%)} = \left[100 - \frac{4 \text{ h hCG (no addition)} - 4 \text{ h hCG (+ additions)}}{4 \text{ h hCG (no addition)}} \right] \times 100$$

Fig. 6. Cross-linking of ^{125}I -hCG and ^{125}I -DG-hCG to receptors on MA-10 cells

MA-10 cells prepared for steroidogenesis as in Fig. 2 were incubated in triplicate with labelled hormones (1.5 ng per well; 250 000 c.p.m.), in the presence or absence of 1 μg of unlabelled preparations, for 3 h at 37 °C in 500 μl . After incubation, cells were washed several times with cold medium (4 °C) to remove free hormones and finally with 0.05 M-phosphate buffer, pH 7.5. The cells adhering to the plate were then treated with 270 μl of this buffer plus 30 μl of cross-linking agent disuccinimidyl suberate (DSS) dissolved in dimethyl sulphoxide to provide a final concentration of 5 mM-DSS. After 15 min at 22 °C, buffer was removed and the cells were rinsed several times with 0.05 M-Tris/HCl, pH 7.5. The cell mat was finally exposed to 4% SDS to extract the membranes. Supernatants were used for SDS/polyacrylamide-gel electrophoresis analysis. In lanes 1–3, cells were exposed to ^{125}I -DG-hCG and lanes 4–6 contain ^{125}I -hCG-treated cells. In lanes 1 and 4, cells each containing 1 μg of unlabelled DG-hCG or hCG were cross-linked. Lanes 2 and 5 were run with dithiothreitol (reduced samples). Lanes 3 and 6 are unreduced samples. The total specific binding in this experiment was 5–7%. The positions of M_r markers are shown (from top to bottom): myosin, β -galactosidase, phosphorylase *b*, bovine serum albumin, carbonic anhydrase, trypsin inhibitor and lysozyme. Bands below M_r 66 200 in lanes 2–6 are due to labelled ligands/ α -subunits.

series of experiments wherein the addition of the DG-hormone was delayed (Fig. 5). In this experiment (Fig. 5a), cells were first exposed to hCG (1 ng/well) at zero time, and then DG-hCG (100 ng/well) was added at various times thereafter in each set of duplicates or triplicates. Control wells in this series received hCG alone. The entire incubation was terminated after 4 h and the total progesterone accumulated in the medium was measured. Figs. 5a and 5c show the time course of

progesterone accumulation for hCG (continuous lines). The amount of steroid secretion was related to the time of addition of DG-hCG, and maximum (nearly 100%) inhibition was produced when it was added at zero time (broken lines). From these responses the percentage inhibition versus delay of DG-hCG addition was calculated (Fig. 5b). Interestingly, delaying DG-hCG addition for up to about 1 h after initiating the action of hCG could still result in a 50% decrease in progesterone accumulation. In another series, this effect was compared with the effect of addition of affinity-purified antibodies to hCG in terminating hormone action (Figs. 5c and 5d). The neutralizing action of the antibody resulted in 50% inhibition of steroid secretion even after a 2 h delay.

Binding of hCG/DG-hCG to receptors on MA-10 cells

Following affinity cross-linking and autoradiography, both ^{125}I -hCG and ^{125}I -DG-hCG showed very similar labelling patterns (Fig. 6), except for a small but distinct difference in apparent M_r of the hormone–receptor complex. In analytical SDS/polyacrylamide-gel electrophoresis, the cross-linked ^{125}I -hCG–receptor complex under non-reduced conditions migrated with an M_r of about 120 000 (Fig. 6, lane 6). Upon reduction to break the S–S bonds this band persisted, but a new component with faster mobility indicating a lower M_r was also generated (Fig. 6, lane 5). This may have been due to the absence of cross-linking of the β -subunit of the hormone in the complex, which may have been eliminated following reduction by dithiothreitol. As the β -subunit is not usually labelled during iodination of the hormone [3], this component does not appear on the autoradiogram. In the presence of 1 μg of unlabelled hCG or DG-hCG during the formation of hormone–receptor complex, there were no bands in the 120 000- M_r region (Fig. 6, lanes 1 and 4). Corresponding data (unreduced and reduced) for ^{125}I -DG-hCG cross-linked receptor are shown in lanes 2 and 3. The banding pattern of the complex was similar to that produced by hCG, except that the migration of the corresponding bands was faster. This was consistent with the decrease in the M_r of hCG upon deglycosylation (hCG, theoretical value 37 000; DG-hCG, 27 200) [15].

DISCUSSION

This work represents a complete evaluation of steroidogenic and other hormonal properties of chemically deglycosylated hormones LH and hCG in mouse Leydig tumour cells and normal cells. Three major parameters of hormone action, namely receptor binding, cyclic AMP accumulation and steroidogenesis, have been scrutinized. In every study examined thus far, for chemically deglycosylated oLH [2,19,24], bovine LH [12] hCG [7,15,16,17,18], oFSH [30] or hFSH [31], or for enzymically deglycosylated hCG [12], a drastic reduction in the gonadal cyclic AMP response have been observed *in vitro*. However, variable steroidogenic effects have been noted. Most studies *in vitro*, utilizing enzymes for dispersion and pooled cells from normal rat testis/ovary, generally agree on the low steroidogenic activities of DG-LH and DG-hCG [2,7,9,12,15,16,19,24,32] but differ in quantitative terms of efficacy, i.e. the ability to generate the same maximum response as the hormone. Contrary to all, Cole *et al.* [23] found that oLH and hLH, deglycosylated by endoglycosidase F treatment, retained

about 50% activity, and concluded that carbohydrate does not play a significant part in hormone action. In view of the resistance of several pituitary and chorionic gonadotropins [33] and pituitary thyrotropins [34,35] to the action of *N*-glycanase and/or endoglycosidase F, it may be suspected that adequate removal of sugars was not obtained in the study by Cole *et al.* [23]. Thus incompletely digested LH preparations may not show significant decreases in steroidogenic potency [23]. Investigations on mouse and human TSH [34,35] and other glycoproteins [36] suggest that not all of the *N*-linked oligosaccharide chains present at various sites in the polypeptide chains may be accessible for enzymic deglycosylations. For human TSH, effective deglycosylation by enzymes could be achieved only after extensive denaturation [35]. If enzymic treatment did not effectively attack the *N*-glycosylation at position 56 of the α -subunit of lutropin, a site playing a key role in signal transduction [13,14], then significant biological activity would be retained in enzymically deglycosylated preparations.

As some of apparent discrepancies noted in the literature with regard to steroidogenic potency and efficacy of DG-LH/DG-hCG may be directly or indirectly related to the problem of the cellular heterogeneity present in normal male [37] and female [38] gonadal cells, we decided to investigate the use of clonal cell lines. Because of its clonal nature, the state of the receptor-effector system can be presumed to be the same in all cells [39]. It can be concluded that in mouse Leydig tumour cells, hormone binding and steroidogenesis are completely dissociated, but in cells of normal testis containing a mixed population, some androgen synthesis, but not second messenger generation, occurs (Figs. 1 and 2a). Although cyclic AMP levels were not estimated in this study (Fig. 2a) with MA-10 cells, others using similar tumour cells (MLTC-1) [17] found DG-hCG to be inactive. That cyclic AMP was not produced in adequate amounts is also clear from the failure of DG-LH/DG-hCG to cause changes in shape of the tumour cells (Fig. 3). These typical rounding phenomena [28] occurring in the presence of LH/hCG are directly attributable to cyclic AMP. The cyclic AMP second messenger, if produced, is apparently not compartmentalized within the steroidogenic cell [7]. Inhibition of phosphodiesterase could enhance the steroidogenic activity of LH and hCG but not of DG-LH and DG-hCG, as shown by experiments with MA-10 cells incubated according to conditions in Fig. 2 but in the presence of 0.05 mM-isobutylmethylxanthine (results not shown). In MA-10 cells, the extremely weak steroidogenic activity of chemically deglycosylated LH and hCG (Fig. 2a) was lower than the activity of the isolated α - and β -subunits of the native hormones. Since the isolated subunits have low affinity for the LH receptor [1-4], the discordant effects of DG-LH/DG-hCG, which bind tightly to the receptor, on steroidogenic properties are readily evident.

Among the models investigated thus far, the mouse Leydig tumour cells (MA-10 cells) are the best system to reveal the potent antagonistic behaviour of DG-LH and DG-hCG. Steroidogenesis was completely inhibited by DG-LH/DG-hCG (Fig. 4). By varying the order of addition of agonist/antagonist (Fig. 5), it could be shown that an excess of DG-hCG was effective in displacing prebound hCG on MA-10 cells and that DG-hCG prevented further binding of the agonist beyond the specified intervals selected in the study. Thus in this

experiment, whatever the initial hormone signal that was transduced before antagonist addition continued unabated for the remainder of the 4 h duration. The net effect of the presence of the antagonist was similar to the presence of a neutralizing gonadotropin antibody, but the mechanisms of inhibition of steroidogenesis are clearly different. Both of these preparations must have been capable of dislodging hCG bound to the receptor, because removal of membrane-bound hCG/LH in MA-10 cells [40] as well as in minced mouse Leydig cell tumours [41] has been shown to result in the cessation of further steroidogenesis. Some factors which may contribute to the uniformity of the antagonistic effects of DG-LH/DG-hCG in MA-10 cells are as follows. (1) In the MA-10 cells, maximal stimulation of progesterone synthesis occurs when approx 70% of the receptors are occupied [42], whereas in normal (rat) Leydig cells (perhaps in other species also), only 1% of the total LH/hCG receptors need to be occupied to sustain maximal steroidogenesis [39]. (2) There is some suggestion that in normal rat Leydig cells there may be more than one signal transduction mechanism for LH [22,43] and that this may operate differently for DG-LH [22]. LH and hCG are apparently unable to stimulate the inositol phosphate/diacylglycerol pathway in MA-10 cells [44], suggesting that cyclic AMP is an obligatory mediator of steroidogenesis by hCG in MA-10 cells.

The demonstration in Fig. 6 that both hCG and DG-hCG apparently bind to the same membrane component(s) (receptors), along with the potent antagonistic effects (Figs. 4 and 5), provide convincing evidence that the blockade of hormone action occurs at the level of the receptor. The M_r of the receptor component, after subtracting the contribution of the ligand in the complex (hCG/DG-hCG), is approx. 90000 (Fig. 6), a value which is very similar to that reported by others for MA-10 cells [45]. The slightly lower value obtained after reduction is also similar to previous observations [45] and could arise from the dissociation of non-cross-linked β -subunit from the complex.

It has been suggested that the loss of antennary carbohydrate units in glycoprotein hormones leads to the formation of unproductive hormone-receptor complex [8]. Certain antibodies [46-48] may convert a poor stimulator such as DG-hCG/DG-LH into a state compatible for complete signal transduction following appropriate incubations *in vitro*, resulting in the full hormonal response. Thus the presence of these antibodies may have substituted for the lost carbohydrate residues and led to a reorientation of the hormone-receptor complex [8]. In these instances there is complete revival of steroidogenesis by DG-LH and DG-hCG in the presence of respective LH and hCG antibodies in MA-10 cells [48], as well as all the associated changes in cell shape (Fig. 3).

In conclusion, the present studies with mouse Leydig tumour cells have established that gonadotropin receptor binding and intracellular activation mechanisms including steroidogenesis can be completely dissociated, and the presence of antennary carbohydrate residues is obligatory for hormone signal transduction.

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