Inhibition of Adenylyl Cyclase Activity in Rat Corpora Luteal Tissue by Glycopeptides of Human Chorionic Gonadotropin and the α-Subunit of Human Chorionic Gonadotropin†

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ABSTRACT: Indirect evidence has indicated that the carbohydrate moieties of the glycoprotein hormones are involved in the activation of the receptor–adenylyl cyclase system of reproductive tissues. In the present study, we have isolated the glycopeptides (GP) from human chorionic gonadotropin (hCG), the α-subunit of hCG, fetuin, and bovine γ-globulin (bγG). These along with a number of synthetic oligosaccharides were tested for their ability to inhibit adenylyl cyclase (AC). There was less than 0.001% cross-reactivity of the GP from hCG, hCGα, fetuin, and bγG when tested in a double-antibody hCG radioimmunoassay or rat corpora lutea radioreceptor assay. The GP of fetuin, bγG, and the synthetic oligosaccharides did not inhibit AC activity of 2000*g* corpora lutea membranes when coincubated with 100 ng of hCG/mL (ED_{50}). However, when the GP of hCG and hCGα were included with intact hCG, there was a dose-related inhibition. Inhibition of cyclase activity was enhanced when the hCG GP were desialylated. This occurred without a change in the lag time of hCG activation which was calculated to be 1–1.5 min. Changing the concentration of ATP and Mg^{2+} did not affect the inhibitory effects of the hCGα GP on hCG-stimulated AC activity. Inhibition by hCG GP followed uncompetitive kinetics. The inhibition by the GP of hCG seems to be restricted to the LH/hCG-stimulatable AC system because the same dosage of hCG GP which inhibited the rat luteal AC system did not have any effect on the rat hepatocyte AC system when coincubated with glucagon or on NaF-stimulated activity in luteal membranes. These results suggest that a membrane lectin may be involved in the regulation of luteal cell hCG-stimulated adenylyl cyclase. We further speculate that receptor and lectin cross-linking may be involved in microaggregation, cyclase stimulation, and internalization.

The mechanism of action of the glycoprotein hormones, including human chorionic gonadotropin (hCG),[1] has been studied extensively (Moyle, 1980; Ryan, 1982; Bahl & Kalyan, 1983). It is known, for example, that binding of hCG to the LH/hCG receptor and subsequent full activation of the adenylyl cyclase (AC) system in vivo and/or in vitro require an intact hormone or one in which only the sialic acid moieties of the carbohydrate chains have been removed. Progressive deglycosylation by enzymatic or chemical means leads to decreased AC activation with a parallel decrease in cellular response but without loss of receptor binding (Chen et al., 1982; Manjunath & Sairam, 1982; Sairam & Manjunath, 1983; Kalyan & Bahl, 1983; Moyle et al., 1975). In addition, the deglycosylated preparations effectively compete with the intact hCG molecule for receptor sites and inhibit biological activity (Keutmann et al., 1983; Sairam & Manjunath, 1983).

We have studied this phenomenon further utilizing the glycopeptides (GP) prepared from hCG, the α-subunit of hCG (hCGα), fetuin, and bovine γ-immunoglobulin (bγG). The GP of fetuin and bγG are generally similar in composition to those of hCG, but there are some differences (Kornfeld & Kornfeld, 1976; Baezinger & Fiete, 1979). In addition, we have investigated the ability of a variety of chemically synthesized oligosaccharides (Arnarp & Lönngren, 1981; Arnarp et al., 1982; Lönn & Lönngren, 1983) to inhibit AC activity in luteal tissue and liver preparations.

Experimental Procedures

Preparation of Glycopeptides. Twenty-five milligrams of highly purified hCG (CR-121, 13 000 IU/mg; a gift of Dr. Robert Canfield, Columbia University) or hCGα (K1182α) was reduced and carboxymethylated (RCXM) according to the procedure of Crestfield et al. (1963). Briefly, to a 20-mL brown glass flask were added in sequence the hormone, 3.61 g of ultrapure guanidine hydrochloride (Schwarz/Mann), 300 μL of 50 mg/mL disodium ethylenediaminetetraacetate (EDTA, Sigma), 3 mL of 40 mM Tris buffer, pH 8.6 (Sigma), 1 mL of Cleland's reagent (0.2 M, Sigma), 3.2 mL of distilled water, and 4.5 mL of 8 M Gdn·HCl with 0.2% EDTA. All solutions were gassed with N_2 before addition. The mixture was stirred for 4 h under a N_2 atmosphere at room temperature. At the end of 4 h, 1 mL of 0.4 M iodoacetate (Sigma) in 1 N NaOH was added and the incubation continued for an additional 15 min. The RCXM gonadotropins were then applied to a 2.6 × 60 cm column of Sephadex G-75 (Pharmacia Fine Chemicals) previously wrapped in aluminum foil and equilibrated in 0.1 M ammonium bicarbonate buffer, pH 8.6. The RCXM hCG and RCXM hCGα peaks were determined by monitoring the 280-nm optical density. The pertinent fractions were then pooled and lyophilized. They

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[1] Abbreviations: hCG, human chorionic gonadotropin (choriogonadotropin); AC, adenylyl cyclase; hCGα, α-subunit of human chorionic gonadotropin; bγG, bovine γ-immunoglobulin; RCXM, reduction and carboxymethylation; GP, glycopeptide(s); LH, luteinizing hormone; Con A, concanavalin A; RIA, radioimmunoassay; Gdn·HCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; RRA, radioreceptor assay; BSA, bovine serum albumin; PMSG, pregnant mares serum gonadotropin.

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were then dissolved in phosphate-buffered saline (PBS), pH 7.5, at a concentration of 1 mg/mL prior to digestion with trypsin (189 units/mg; Millipore Corp.) and Pronase (106 units/mg from *Streptococcus griseus*, Calbiochem-Behring Corp.). Digestion was accomplished with 10 μg of trypsin/mL for 1 h followed by the addition of 50 μg of Pronase/mL during the second h and an additional 50 μg of Pronase/mL the third hour at 37 °C with shaking. The digestion was monitored by the release of ^{125}I from a parallel coincubation of ^{125}I-hCG. After the hydrolysis was judged to be complete, the digest was mixed with concanavalin A coupled to Sepharose 4B (Sigma) for 20 min at 4 °C. The slurry was then poured into a column, allowed to settle, and washed with PBS, pH 7.5. The bound glycopeptide fractions[2] were then eluted off the column (1 × 10 cm) with 0.1 M borate buffer, pH 6.0. The 4-mL fractions collected were monitored for sialic acid containing GP by the fluorometric thiobarbituric acid method of Hammond & Papermaster (1976). The eluted fractions which showed the highest concentration of sialic acid were pooled, lyophilized, dissolved in 15 mL of distilled water, and applied to a 3 × 30 cm Bio-Gel P2 column (Bio-Rad) previously equilibrated in 0.1 M borate buffer, pH 6.0. The fractions were again monitored for sialic acid content. The sialic acid containing fractions were then subjected to ultrafiltration in an Amicon UM-05 filter (M_r 500 cutoff) in order to concentrate and desalt the GP. The presence of residual proteolytic activity was checked in a protease assay which utilizes ^{125}I-glucagon as the substrate (Jusic et al., 1976). No proteolytic activity was detected in the hCG or hCGα GP fractions. Recoveries based on the sialic acid content of the GP of hCG and hCGα were 61% or 5.03 mg and 70% or 5.7 mg, respectively.[3]

In some experiments, the sialic acid was removed from the hCG GP by treatment with neuraminidase. The hCG GP were incubated with neuraminidase from *Clostridium perfringes* (Sigma type X-A) coupled to agarose for 20 min at 37 °C in 0.1 M ammonium acetate buffer, pH 5.0. The desialylated GP was isolated from the immobilized neuraminidase by centrifugation, lyophilized, and reconstituted to a final concentration of 1 mg/mL.

The asparagine-linked GP of fetuin, the major glycoprotein in fetal calf serum, were isolated by the procedure of Baenziger & Fiete (1979). Type III fetuin was purchased from Sigma and was used without further purification. However, during the isolation procedure, we determined that a small but measurable amount of protease activity coeluted with the fetuin GP fraction. This protease contamination, due to the Pronase treatment, may have been acceptable to Baenziger & Fiete (1979) since their main concern was with the determination of the structure of the fetuin oligosaccharide. In our studies, we could not tolerate any protease contamination because Richert & Ryan (1977) have shown that very low concentrations of various proteases stimulate AC activity. This contamination may have the undesirable effect of masking any effect which the GP of fetuin may have on the luteal AC system. For this reason, we utilized as a final purification step a 2.5 × 90 cm column of Bio-Gel P-6 (Bio-Rad) equilibrated with 0.2 M ammonium formate and 30% acetonitrile, pH 6.0. The protease and sialic acid contents were monitored as described previously. We also monitored the hexose content of the column effluent by the phenol–sulfuric acid assay for hexoses of Dubois et al. (1956). After identification of the GP fractions, they were pooled and lyophilized for subsequent testing.

Bovine IγG (Schwarz/Mann) was reduced and carboxymethylated according to Crestfield et al. (1963) with the following modifications. The RCXM bγG was dialyzed extensively first against deionized water, then against distilled water, and finally against PBS, pH 8.0. The dialyzed RCXM bγG was then digested, under toluene, with 2 additions of 1% w/w trypsin 2 h apart. After the 4-h incubation, 1% w/w Pronase was added and the reaction allowed to proceed for an additional 4 h. The pH was adjusted to 8, an additional 1% w/w Pronase was added, and incubation was continued overnight. The digest was then lyophilized, reconstituted, and applied to a 2.5 × 90 cm Bio-Gel P-6 column equilibrated with 0.2 M ammonium formate and 30% acetonitrile, pH 6.0. The purification was monitored with the phenol–sulfuric acid assay (Dubois et al., 1956) for neutral sugars because of the absence of sialic acid residues in the carbohydrate structure of the oligosaccharides of bγG (Kornfeld & Kornfeld, 1976).

The chemically synthesized oligosaccharides were a gift of Dr. J. L. Lönngren of Pharmacia Fine Chemicals, Sweden, and H. L. Lönn of Stockholm University. They were classified according to the number and kind of sugar residues as shown in Chart I. The synthesis and purification of these complex oligosaccharides have been described (Arnarp & Lönngren, 1981; Arnarp et al., 1982; Lönn & Lönngren, 1983).

Adenylyl Cyclase and Radioreceptor Assays. The various GP and oligosaccharides were then tested for activity in a hCG radioreceptor assay (RRA; Lee & Ryan, 1973) and an adenylyl cyclase assay utilizing 2000*g* corpora lutea or liver membrane fractions (McIlroy & Ryan, 1980) prepared from 30-day-old PMSG-hCG primed immature rats (Holtzmann, Madison, WI; Lee & Ryan, 1973).

Investigations of the effect of hCGα GP on the kinetics of adenylyl cyclase activity were made by using increasing doses of ATP and a constant 2 mM excess of Mg^{2+}. These data were analyzed by Lineweaver–Burk and Dixon plots and least-squares regression analysis (Batson, 1956).

Radioimmunoassays. Some of the GP were tested for immunological cross-reactivity in a double-antibody hCG radioimmunoassay (RIA) as described by Faiman et al. (1967). The first antibody against native hCG was generated in rabbits and was used at a final dilution of 1:3 000 000. The sensitivity of the assay was 0.02 ng/tube.

Results

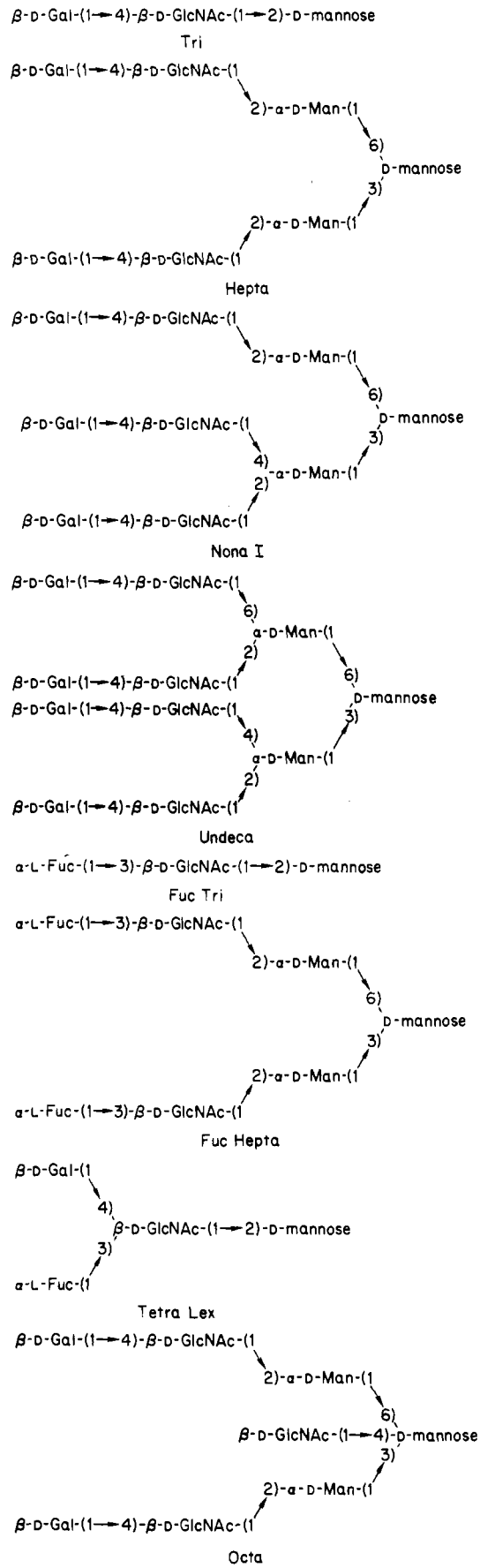
There was no cross-reactivity of the GP isolated from hCG, hCGα, bγG, and fetuin when they were tested in a hCG RIA or RRA (data not shown). For every GP tested, the relative potency was significantly less than 0.001%, as no activity was found at 200 μg of GP/mL, the highest concentration tested.

To rule out the possibility that no interaction was seen in the equilibrium RRA due to a very high dissociation rate, an association rate experiment in which 100 μg of hCG GP/mL

[2] The asparagine-linked glycopeptides, which contain mannose, bind to Con A while the serine-linked glycopeptides, which lack mannose, do not bind.

[3] Amino acid anaylsis (performed by Dr. Henry Keutmann, Massachusetts General Hospital) indicated that the glycopeptide fraction which was isolated from hCG contained the following in mole percent: lysine, 2.5; histidine, 3.5; arginine, 9.4; aspartic acid, 15.7; threonine, 13.4; serine, 4.3; glutamic acid, 5.5; proline, 2.2; glycine, 4.4; alanine, 4.1; valine, 7.3; isoleucine, 7.0; leucine, 4.1; tyrosine, 2.0; phenylalanine, 1.1; and *N*-acetylglucosamine, 13.4. Note that most of these residues fall near the Asn-X-Thr sequences of hCGα and -β. The molecular weight of the glycopeptides of hCG was estimated to be 4000 by gel filtration. The average molecular weight of the N-linked glycopeptides of hCG has been calculated to be 2577. This difference of approximately 1400 daltons is probably due in part to amino acids still attached to the glycopeptides as indicated by the amino acid analysis. In addition, the hydrodynamic behavior of carbohydrate chains under gel filtration is such that they behave as larger molecular weight species.

Chart I



was coincubated with 2 ng of ^{125}I-hCG/mL was performed. As shown in Figure 1, the addition of the hCG GP did not change the association rate constant (4.314×10^8 and 4.289 $\times 10^8$ M^{-1} min^{-1} for hCG and hCG + hCG GP, respectively). This further demonstrates that the carbohydrate moieties of hCG are not required for binding to the LH/hCG receptor.

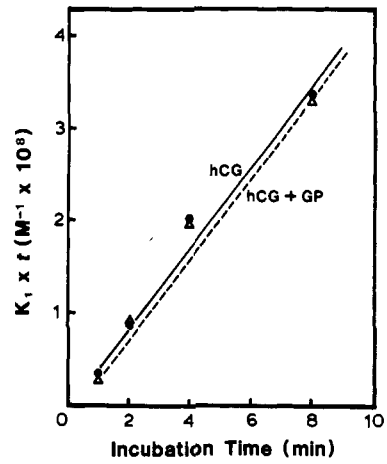


FIGURE 1: Association rate experiment for ^{125}I-hCG in the absence (●) or presence (△) of 100 μg of hCG glycopeptide/mL. Assay conditions were the same as for an equilibrium binding assay as described by Lee & Ryan (1973) with the following modifications. The incubations were performed at 25 °C with 4.3×10^{-10} M (2.0 ng/mL) ^{125}I-hCG for 1–8 min following which 3 mL of ice-cold Tris buffer with 0.1% BSA was added to the incubation vial. At the indicated times, the suspension was immediately filtered with suction through a Metricel filter (0.45-μm pore size) previously wet with 4% BSA in 40 mM Tris buffer, pH 7.4. The adsorbed material was washed twice with 3 mL of buffer, and the radioactivity was measured. The initial reaction rate was plotted according to $K_{+1}T = (H - R)^{-1} - \ln [R(H - X)H^{-1}(R - X)^{-1}]$. The slope of the graph ($K_{+1}T$) on the ordinate vs. T on the abscissa gives the association rate constant (K_{+1}).

We investigated the effects of the hCG GP on the time course of hCG stimulation of adenylyl cyclase. This was studied both with the native GP and also with GP in which the terminal sialic acids had been removed with neuraminidase. These results are shown in panels A and B of Figure 2. The time course of AC activation was linear for both GP over the 32-min observation period. The addition of 20 μg/mL either of the asialo-GP or of the native GP concurrently with 100 ng of hCG/mL did not significantly inhibit the hCG-stimulatable AC activity when compared to hCG without glycopeptides. However, when 200 μg of hCG GP or the asialo-GP/mL was included in the assay, there was a significant inhibition of hCG-stimulatable AC activity. The inhibition of the hCG activity by the GP of hCG was more pronounced when the GP was desialylated (compared panels A and B of Figure 2). In addition, there was no difference in the lag time of AC activation whether or not the hCG GP were coincubated with hCG. The lag time was calculated to be 1–1.5 min in all cases which is normal under the present assay conditions.

To determine if the inhibitory response of the hCG GP on AC activity was specific for the LH/hCG-stimulatable AC system, the GP was tested in a glucagon-stimulatable AC system which utilized hepatocyte 2000g membrane pellets. As shown in Figure 3A, the same dosages of the intact or asialo-GP which exhibited a significant inhibitory effect on the luteal AC had no significant effect on the hepatocyte AC system (compare panels A and B of Figure 3).

Panel C of Figure 3 illustrates the dose–response inhibition by the asparagine-linked GP of hCGα. In contrast to previous experiments, the hCGα GP were preincubated at room temperature with the membrane preparations for 10 min prior to the addition of hCG and the reaction mixture. A dose-related inhibition was demonstrated, and maximum inhibition was achieved at 200 μg/mL hCGα GP. This resulted in a 77%

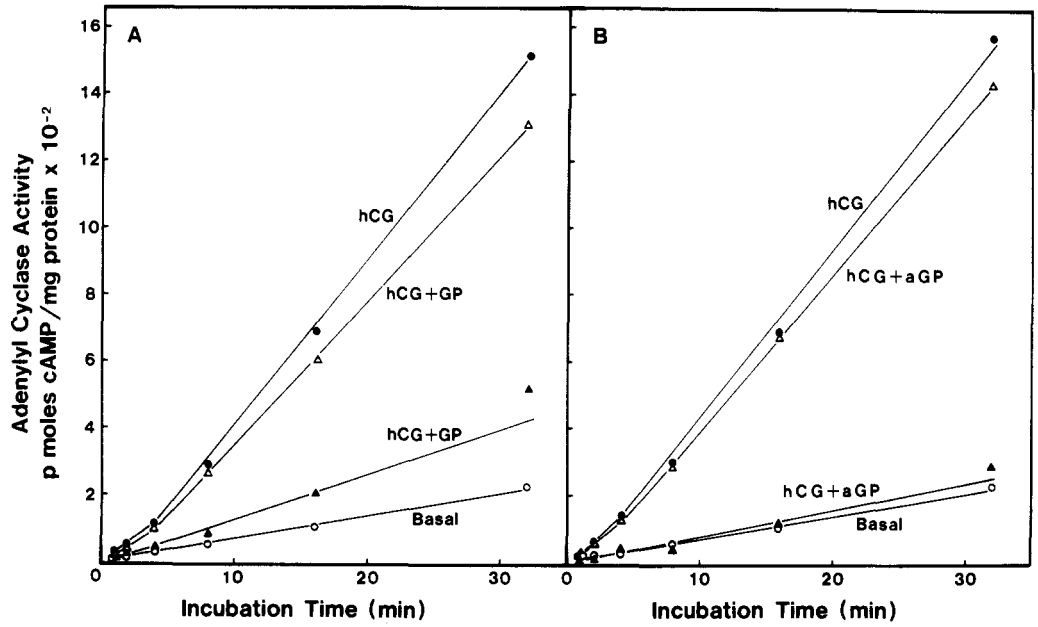


FIGURE 2: Time course (1–32 min) of hCG-stimulatable adenylyl cyclase activity of a 2000*g* rat luteal membrane preparation in response to 100 ng of hCG/mL (●), hCG plus 20 (△) or 200 (▲) μg of hCG glycopeptide/mL (A), hCG plus 20 (△) or 200 (▲) μg of desialylated glycopeptide/mL (B), and basal (○). The desialylated hCG glycopeptide was prepared as described under Experimental Procedures. The symbols represent the mean of two separate experiments performed in triplicate.

inhibition when compared to the hCG-stimulatable AC activity without any GP. Fifty micrograms per milliliter hCGα GP when coincubated with native hCG resulted in a 43% inhibition of cyclase activity. Similar results were obtained with the GP of hCG, and desialylation of the hCG GP enhanced the inhibitor activity (Figure 3B).

In experiments with the GP of either hCG or hCGα, we inconsistently observed that they decreased basal activity. In order to determine if the decrease in basal activity was due to a nonspecific effect of the GP on one of the constituents of the assay mixture, we tested the effects of various ATP:Mg^{2+} ratios. The results of this experiment are shown in Figure 4. In the cases illustrated, there was a depression of both the basal activity and also the hCG-stimulatable AC activity when coincubated with 100 μg/mL GP. The left-hand panel of Figure 4 illustrates the inhibition that is seen with the concentrations of ATP and Mg^{2+} normally employed in this laboratory. There was no change in the degree of inhibition of the hCG-stimulatable AC activity when coincubated with the hCGα GP at different ATP:Mg^{2+} ratios. However, when the basal activities with and without 100 μg of hCGα GP/mL were compared, there was a greater inhibition of basal activity when the ration of ATP to Mg^{2+} was 2:9. At this time, we are unable to explain the inconsistent inhibition of the basal AC activity by the GP of hCG or hCGα. The GP had no effect on NaF-stimulated activity (data not shown).

Results obtained when the GP of bγG, fetuin, and the synthetic oligosaccharides Tri, Hepta, Nona, Undeca, Fuc Tri, Fuc Hepta, Tetra Lex, and Octa were coincubated with 100 ng of hCG/mL showed no inhibition of luteal AC activity by any of these substances. The lack of inhibition was noted even if the GP and the synthetic oligosaccharides were preincubated with the luteal membranes before the addition of hCG and the reaction mixture. The highest dose tested was 200 μg/mL (data not shown). In addition, we also tested the effect of the individual sugars fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, mannose, glucose, and galactose on their ability to inhibit the luteal AC system at a dose of 200 μg/mL and found them to be without effect (data not shown).

Table I: Summary of Kinetic Parameters of the Interaction of Adenylyl Cyclase Enzyme, Relative to ATP, under Various Treatments

	V_{max} [pmol min^{-1} (mg of protein)$^{-1}$]	K_m (mM)
30 ng of hCG/mL[a]	14.05	0.45
100 ng of hCG/mL	32.10 ± 4.33 (3)[b]	0.46 ± 0.11 (3)
100 ng of hCG/mL + 100 μg of hCG GP/mL	22.88	0.36
100 ng of hCG/mL + 200 μg of hCGα GP/mL	10.15	0.15
5 μg of protein[c]	29.94	0.24
10 μg of protein	57.54	0.52
30 μg of protein	295.0	2.86

[a] The concentration of protein per tube in these experiments was 15 μg. [b] The numbers in parentheses represent individual experiments performed in triplicate. [c] The V_{max} and K_m values for 5, 15, and 30 μg of protein were obtained with a different membrane preparation than those reported above. The concentration of hCG at each level of protein was 100 ng/mL.

The interaction between the AC enzyme, hCG, and hCGα GP was studied as a function of ATP concentration with a constant 2 mM excess of Mg^{2+} over ATP. The double-reciprocal plot in Figure 5 indicates that the GP caused a decrease in both the K_m and the V_{max} for the reaction. Although the slopes of the plots for hCG and hCG + hCGα GP appear nonparallel, statistical analysis indicates they they do not differ.

To study this phenomenon further, the amount of active AC enzyme was varied by using different amounts of membrane protein as the source of enzyme. The reduction of membrane protein from a 30 μg/mL assay mixture to 5 μg/mL caused a decrease in both K_m and V_{max}, and the slopes of the response parameters did not differ significantly. Results of these experiments are given in Table I. Changing the concentration of hormone while maintaining a constant amount of enzyme (15 μg of protein per tube) changed V_{max} without altering the K_m (Table I).

Discussion

The importance of this study lies in the demonstration of a biological effect of the GP moieties of hCG and hCGα: the

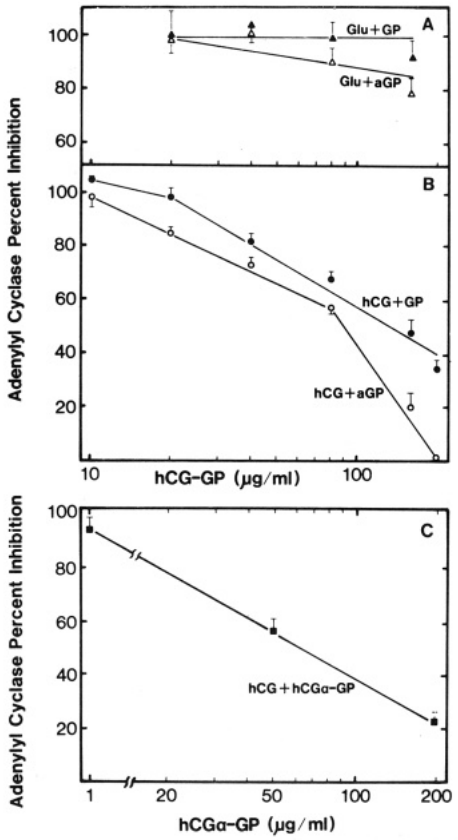


FIGURE 3: Effect of increasing concentrations of hCG glycopeptide (▲, ●), hCG desialylated glycopeptide (△, ○), and hCGα glycopeptides (■) on liver (glucagon, 0.2 μM; panel A) and ovarian luteal membrane (hCG, 100 ng/mL; panels B and C) adenylyl cyclase activity. The concentrations of glucagon and hCG were kept constant with increasing concentrations of the hCG or hCGα glycopeptides. Incubations were started by the addition of the respective pellet (equivalent to 1 mg/mL protein) except for the hCGα glycopeptides (panel C) in which they were preincubated with the luteal membrane pellet for 10 min at room temperature prior to the addition of the reaction mixture. Incubations were terminated after 20 min at 30 °C by the addition of 100 μL of 40 mM ATP, 12.5 mM cyclic AMP with 10000 cpm of [^{3}H]cAMP, and 1% sodium dodecyl sulfate followed by heating at 100 °C for 3 min. Results are expressed as percent inhibition (100% is taken to be the adenylyl cyclase stimulability in the absence of hCG glycopeptide after the base line has been subtracted) and represent three separate experiments performed in triplicate.

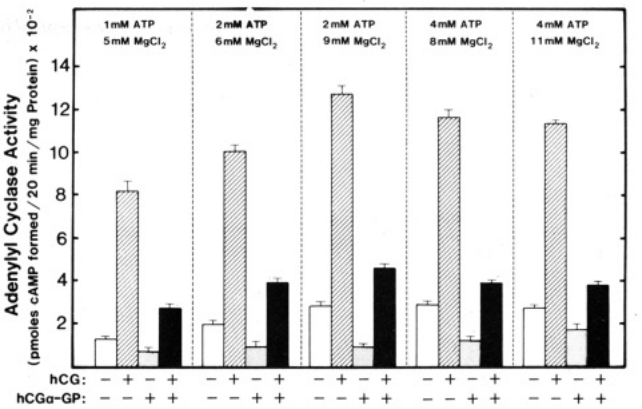


FIGURE 4: Effect of different ratios of ATP to $MgCl_2$ on the ability of the glycopeptides of hCGα to inhibit the basal and hormone-stimulatable adenylyl cyclase system of rat ovarian luteal membranes. The concentration of the hCGα GP was kept constant at 100 μg/mL, and the GP were preincubated where indicated with the luteal membranes for 10 min at room temperature before addition of the reaction mixture. The concentration of hCG (100 ng/mL) was also kept constant and was included in the assay where indicated. The left-most panel (1 mM ATP:5 mM $MgCl_2$) represents the concentrations of ATP and $MgCl_2$ which are normally used in our assay system. The bars and horizontal lines represent the mean ± SEM of triplicate determinations. The symbols on the abscissa indicate the presence (+) or absence (−) of hCG (100 ng/mL) and hCGα glycopeptides.

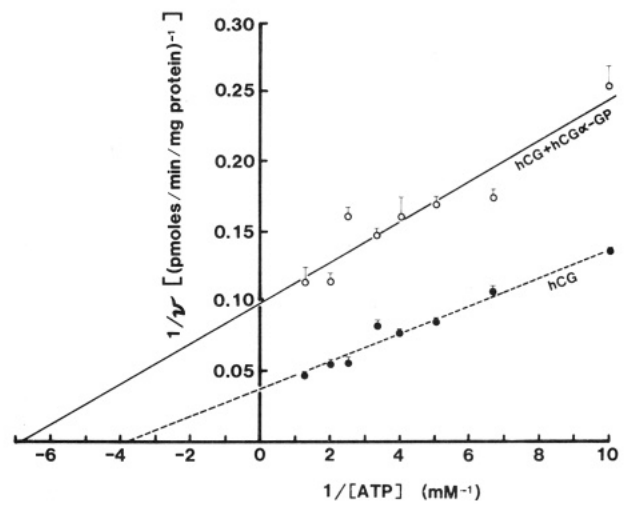


FIGURE 5: Double-reciprocal plots of the effects of hCG (●) and hCG + hCGα GP (○) on hormone-stimulatable adenylyl cyclase activity at various ATP concentrations. The adenylyl cyclase activity was measured as described under Experimental Procedures and was performed at the indicated concentrations of ATP in the presence of a 2 mM excess of Mg^{2+} over ATP. The regression line was obtained by means of least-squares regression. The hCG and hCGα GP concentrations were kept constant at 100 ng and 200 μg/mL, respectively. Data points are the mean ± SEM of triplicate determinations.

inhibition of adenylyl cyclase activity. As discussed below, this suggests the existence of a carbohydrate binding protein or lectin as an additional component of the luteal cell receptor–AC system.

As shown in Figure 2, our studies indicate that the GP of hCG can successfully compete with the oligosaccharide moieties of the intact hCG molecule and inhibit AC activity without significant inhibition of the binding of hCG to its specific receptor. The hCG-stimulatable AC activity can be inhibited in a dose–response fashion by the GP of either hCG or hCGα as shown in Figures 2 and 3. In addition, the asialo-GP seems to exhibit a somewhat greater potency than the intact GP (Figures 2 and 3). This inhibition is specific in that it is not mimicked by the GP of fetuin or bγG or by a variety of synthetic oligosaccharides, does not occur with the glucagon–hepatocyte AC system, cannot be reversed by varying the assay conditions with respect to the concentrations of ATP and Mg^{2+}, and does not occur with NaF-stimulatable AC activity.

Previous studies which attempted to assign a function to the oligosaccharides of the glycoproteins relied on an indirect approach. These methods involved either desialylation (Tsuruhara et al., 1972a,b), sequential enzymatic treatment to denude the hCG molecule of carbohydrates (Moyle et al., 1975; Channing & Bahl, 1978; Channing et al., 1978f Governman et al., 1982), or chemical methods employing either hydrogen fluoride (Chen et al., 1982; Manjunath & Sairam, 1982; Keutmann et al., 1983; Sairam & Manjunath, 1983; Shimohigashi & Chen, 1982), trifluoromethanesulfonic acid (Kalyan & Bahl, 1983), or periodate oxidation of asialo-hCG (Kalyan et al., 1982). In each of these studies, the conclusion was made that the deglycosylated hCG had an equal or greater affinity for the LH/hCG receptor but showed a reduction or

absence of biological activity in corpora lutea and interstitial cell bioassay systems. Also, the deglycosylated hCG was found to be an inhibitor of native hCG action as judged by the stimulation of cyclic AMP (Chen et al., 1982; Keutmann et al., 1983; Kalyan & Bahl, 1983; Sairam & Manjunath, 1983), progesterone (Kalyan & Bahl, 1983), and testosterone secretion (Sairam & Manjunath, 1983). The loss of biological activity was ascribed to the loss of the carbohydrate residues of hCG.

As an explanation for the effects of deglycosylated hCG and the competition of the GP of hCG for activation of AC by hCG, we hypothesize that the native hormone binds to its membrane receptor through its peptide backbone and to a membrane lectin or lectins through its oligosaccharide side chains. As a corollary, we hypothesize further that the cross-linking of the receptor and lectin(s) is required for the activation of the luteal cell cyclase system and perhaps other phenomena such as microaggregation and internalization (Amsterdam et al., 1980).

The evidence for this hypothesis may be surmised from the results obtained in Figure 5 and Table I in which the kinetics of AC activity were studied. Analysis indicates that the type of inhibition effected by the GP of hCG is uncompetitive. This means that the carbohydrate moieties of hCG and the intact hCG are not binding to the receptor–AC complex at the same time, since, if this were the case, inhibition would be of a competitive nature. This is reflected in the different K_m and V_{max} values which are observed for hCG and hCG + hCGα GP (Table I). We can further speculate that since the GP of hCG do not compete with native hCG in the radioreceptor assay (Figure 1) the binding of the GP is not to the hCG/LH receptor but to another membrane component, such as a lectin. The net effect of the displacement of the native carbohydrate of hCG by those of the GP prepared from hCG is to make less active AC enzyme available. This is seen in Table I where different doses of enzyme, i.e., hormone receptor–AC enzyme complex, also result in an uncompetitive pattern similar to that observed when the hCGα GP are included in the assay. Also, when the protein concentration (AC enzyme) was kept constant and the hCG concentration varied, there was a decrease in the V_{max} but not the K_m at the lower dose of hCG with respect to 100 ng of hCG/mL. This may help to explain the inhibitory nature of the deglycosylated hormone on AC activity. Uncompetitive inhibition has also been demonstrated in bone AC when the effect of Ca^{2+} on bone and bone tumor AC was investigated at various $MgATP^{2-}$ concentrations by Rodan et al. (1980). The uncompetitive inhibition of Ca^{2+} was interpreted to mean that the binding of Ca^{2+} occurs after the $MgATP^{2-}$, yielding a noncatalytic (ESI) complex, thus making less activated enzyme available.

The existence of lectins or carbohydrate binding proteins in plant and vertebrate systems has been known for a long time [see reviews by Barondes (1981) and Olden et al. (1982)]. In mammalian systems, they have been identified in liver plasma membranes (Lee et al., 1983), mouse lymphoma PL 1.3 cell line (Kornfeld et al., 1981), mouse Swiss 3T3 cells, human SL66 foreskin fibroblast cells (Roff et al., 1983), Chinese hamster ovary cells (Hunt, 1982), and other systems. These lectins have been shown to be highly specific for various carbohydrates, in particular, mannose, galactose, and fucose (Lee et al., 1983; Prieels et al., 1978). An interesting aspect of these lectins, as shown by Lee and co-workers (Lee et al., 1983), is the increase in binding affinity to the hepatic galactose/*N*-acetylgalactosamine lectin of oligosaccharides with an increasing number of carbohydrate chains. The binding affinity as measured by the inhibition of binding (I_{50}) of ^{125}I-Tyr-asialo triantennary glycopeptide utilizing freshly isolated rabbit hepatocytes was 1×10^{-3}, 1×10^{-6}, and 5×10^{-9} M for the mono-, bi-, and triantennary oligosaccharides, respectively.

Structural studies (Bahl & Kalyan, 1983) of hCG indicate that there are four asparagine-linked oligosaccharide moieties, two in the β-subunit at asparagines-13 and -30 and two in the α-subunit at asparagines-52 and -78. The β-subunit also contains four serine-linked short oligosaccharide chains at serines-121, -127, -132, and -138. These serine-linked oligosaccharides do not appear to participate in the expression of cellular responses since they are lacking on the LH molecule. The asparagine-linked oligosaccharides are biantennary and are identical except for the presence of one fucose residue attached $\alpha 1 \rightarrow 6$ to the *N*-acetylglucosamine residue of the β-subunit proximal to the asparagine. Therefore, the postulated interaction between the carbohydrate residue(s) in question and the putative lectin(s) could be complex. The data of Shimohigashi & Chen (1982) and Keutmann et al. (1983) indicate that the oligosaccharides are required on both the α- and β-subunits of hCG for activation of luteal AC.

The subtleties of structure and conformation of the glycopeptides and oligosaccharides appear to be crucial for binding to lectins to occur. In our studies, we were perplexed when the GP isolated from bγG, which are quite similar to those of hCGα except for the absence of terminal sialic acids, failed to inhibit the hCG-stimulatable AC activity of corpora lutea membranes. However, the fact that there is a large degree of heterogeneity in the oligosaccharide portion of the γG molecule and that up to 40% of the glycopeptide may lack one galactose residue linked at the *N*-acetylglucosamine residue (Tai et al., 1975) may help to explain the lack of effect of the bγG GP. The lack of effect of the GP of fetuin was not a surprise since they have glycosidic linkages distinctly different from hCG.

Evidence for cross-linking of membrane components as a requirement of activation of biological processes is found in the binding of IgE to mast cell receptors as shown by Ishizaka (1982). In this system, binding of IgE itself does not lead to mast cell degranulation, AC activation, calcium influx, or membrane lipid methylation. However, if the IGE receptor complexes are bridged by divalent antibody to IgE or by its $F(ab')_2$ fragment, then full expression of the above-mentioned events occurs. Expression of biological activity also occurred if the receptors were cross-linked with antibodies to the receptor.

The requirement for large (low micromolar) doses of GP to inhibit hCG activation of luteal adenylyl cyclase deserves comment. First, as pointed out above, the oligosaccharides of hCG have a biantennary structure, and these have a lower affinity for lectin binding than triantennary oligosaccharides [1×10^{-6} vs. 5×10^{-9} M according to Lee et al. (1983)]. Second, native hCG has four asparagine-linked oligosaccharides attached to the peptide core and would at least be tetravalent while the oligosaccharides tested here are single units. Thus, binding of hCG to membrane lectins would be expected to have higher affinity than the isolated oligosaccharides. RCXM hCG inhibit adenylyl cyclase activation by native hCG; however, it is less potent than the isolated glycopeptides (F. O. Calvo and R. J. Ryan, unpublished results). This suggests that steric factors must be involved in the interaction of the oligosaccharides with the membrane.

If cross-linking of the hormone receptor complex with a membrane lectin is not involved with activation of adenylyl

cyclase, then what other mechanisms are possible? Removal of the carbohydrate from hCG may alter the conformation of the peptide portion of the hormone. Previous data (Keutmann et al., 1983) indicated that no cleavage of the peptide chains had occurred and that there was no gross alteration of the circular dichroic spectrum. However, subtle alterations of the reaction of deglycosylated hCG with antisera have been noted. First, the deglycosylated hCG subunits were less potent than their native counterparts in radioimmunoassays using antibodies against native materials (Keutmann et al., 1983). Second, Keutmann et al. (1984) found that an antiserum raised against RCXM hCGα would recognize the N-terminal region of the immunogen but not native hCGα or intact hCG. This antiserum, however, recognized deglycosylated hCGα. Removal of carbohydrate from RCXM hCGα did not enhance its potency in this assay. These immunologic data suggest that subtle conformational changes occur with deglycosylation and these could affect the cyclase activation step. However, these conformational changes would not explain the inhibitory effects of the glycopeptides noted in this report.

Added in Proof

Recently, we have tested the isolated oligosaccharides of hCG, without residual amino acids, for their AC inhibitory activity. They were prepared by Dr. Harold Papkoff (University of California, San Francisco) by hydrazinolysis of hCG and reacetylation of the oligosaccharides. There was a dose-related inhibition of the hCG-stimulatable AC activity, with 200 μg of hCG oligosaccharide/mL effecting a 60% inhibition in cAMP production.

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Registry No. AC, 9012-42-4; ATP, 56-65-5; hCG, 9002-61-3; Tri, 62398-02-1; Hepta, 76542-60-4; Nona I, 84076-06-2; Undeca, 84062-48-6; Fuc Tri, 95274-25-2; Fuc Hepta, 95274-26-3; Tetra Lex, 95274-27-4; Octa, 95274-28-5; Mg, 7439-96-5.

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