Macromolecular photoaffinity labeling of the lutropin receptor on granulosa cells

(human choriogonadotropin/photocrosslinking/photoactivable heterobifunctional reagents)

INHAE JI AND TAE H. JI

Department of Biochemistry, University of Wyoming, Laramie, Wyoming 82071

ABSTRACT   Lutropin, a pituitary hormone, and human choriogonadotropin bind to the same receptors in the ovary and elicit identical responses. A photoactivable derivative of human choriogonadotropin was used to identify the lutropin receptor on porcine granulosa cells. The hormone was condensed with a heterobifunctional reagent, the N-hydroxysuccinimide ester of 4-azidobenzoylglycylglycine, and iodinated. The ^{125}I-labeled hormone (^{125}I-hormone) derivative associated with the same number of receptors as ^{125}I-hormone itself did but with a slightly lower K_a, 2.98 × 10^9 M^{-1} compared with 5.1 × 10^9 M^{-1} for ^{125}I-hormone. The binding could be blocked with untreated hormone or lutropin but not with follitropin, prolactin, insulin, or bovine serum albumin. Its α and β subunits could be crosslinked to produce αβ dimer by photolysis, the extent of crosslinking being dependent upon the reagent concentration used for the derivatization: 22.8% at 50 μM, 37.3% at 100 μM, and 67.2% at 150 μM. When the ^{125}I-hormone derivative bound to the cells was photolyzed for crosslinking and the products resolved by electrophoresis on sodium dodecyl sulfate/polyacrylamide gels under reducing conditions, three new bands of lower electrophoretic mobility appeared in addition to α, β, and αβ bands. Formation of these crosslinked complexes required photolysis and the presence of both cells bearing the receptor and the ^{125}I-hormone derivative. It could be blocked by excess untreated hormone. The three bands correspond to molecular weights, 96,000 ± 6,700, 66,000 ± 4,600, and 63,000 ± 4,400. Because the hormone has a high carbohydrate content and such glycoproteins are known to exhibit anomalous electrophoretic mobilities, these estimates must be tentative.

Lutropin, a glycoprotein hormone composed of two dissimilar subunits, α (18,000 daltons) and β (16,000 daltons), is involved in the regulation of the endocrine and reproductive functions of the gonads (1). It binds to specific surface receptors on ovary cells only as the αβ dimer. Such occupied receptors are thought to play a vital role in triggering a cascade of biological events (2). However, little is known about the chemical identity of the receptors and their association with other membrane components that may be involved in hormone action. Results of affinity column chromatographic studies are not entirely clear; estimates of the molecular weight ranged from 190,000 to 80,000 (2, 3).

Several years ago, macromolecular photoaffinity labeling of surface receptors for polypeptide ligands was developed (4–6). This technique, an alternative to affinity column chromatography, uses photoactivable heterobifunctional reagents. Polypeptide ligands coupled with such reagents under physiological conditions are allowed to bind to their specific receptors which are then crosslinked. Recently it was reported that the 2-nitro-4-azidophenyl derivative of ovine lutropin failed to

crosslink with its receptor (7). We have synthesized a longer and more hydrophilic photoactivable heterobifunctional reagent, the N-hydroxysuccinimide ester of 4-azidobenzoylglycylglycine (ABGG), which reacted with human choriogonadotropin (hCG). This hormone mimics lutropin activities. In this communication, we report the successful photolabeling of specific polypeptides related to the hormone receptor on the surface of granulosa cells.

EXPERIMENTAL PROCEDURES

Materials. The N-hydroxysuccinimide ester of 4-azidobenzoic acid was synthesized as described (8) and 10 mmol of it was dissolved in 60 ml of dioxane. It was added to 10 mmol of glycylglycine in 20 ml of 1 M sodium bicarbonate; the mixture was stirred for 5 hr and then filtered. The filtrate was concentrated to remove dioxane and acidified with hydrochloric acid. The precipitate was collected and recrystallized in ethyl alcohol. The resulting ABGG was esterified with N-hydroxysuccinimide (8). The hCG, batch CR-121, was supplied by the Center for Population Research (National Institute of Child Health and Human Development); its biological potency was 13,450 international units/mg.

Derivatization and Iodination. The ABGG ester was freshly dissolved in dimethyl sulfoxide to a concentration of 100 mM and diluted 1:500 in phosphate-buffered saline (pH 7.4). Fifty microliters was added to 10 μg of hCG in 50 μl of the buffered saline and incubated for 30 min at 25°C. To the solution were added 7 μg of chloramine-T and 1 mCi (1 Ci = 3.7 × 10^{10} becquerels) of sodium [^{125}I]iodide in 10 μl of 0.1 M NaOH; 30 sec later, iodination was stopped by addition of 10 μg of sodium metabisulfite. The derivatized, iodinated hCG (ABGG-^{125}I-hCG) was immediately fractionated on a Sephadex G-75 (superfine) column (0.6 × 15 cm).

Cells and Crosslinking. Medium to large follicles were excised from porcine ovaries, and the granulosa cells were drawn out of the follicles with a syringe. The cells were washed twice with medium 199 and incubated with hCG in medium 199/20% porcine serum for 30 min at 37°C. After two rinsings, the cells were irradiated (UVS-11 lamp) for 3 min (6) and rinsed again. Membranes were extracted in 0.5% Triton X-100/10 mM NaCl/10 mM Tris/1 mM MgCl$_2$, pH 7.5/0.003% phenylmethylsulfonyl fluoride. The extracted supernatant solution was made to 1% in sodium dodecyl sulfate and 2% in mercaptoethanol, boiled, centrifuged at 100,000 × g for 30 min, and electrophoresed on 10% polyacrylamide gel (9).

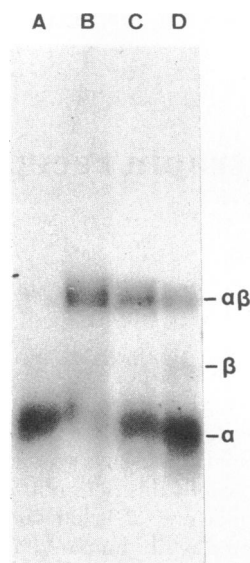Abbreviations: hCG, human choriogonadotropin; ABGG, 4-azidobenzoylglycylglycine.

FIG. 1. Autoradiograph of ^{125}I-labeled ABGG-hCG. Hormone molecules were incubated with varying concentrations of the *N*-hydroxysuccinimide ester of 4-azidobenzoic acid for 30 min at 25°C, iodinated, fractionated, and photolyzed for crosslinking. After solubilization by 1% sodium dodecyl sulfate and 2% mercaptoethanol, the protein components were resolved by electrophoresis. The gel was dried and exposed to x-ray film. Lanes: A and B, derivatized in 150 μM; C, in 100 μM; D, in 50 μM. All samples were photolyzed except that in lane A.

RESULTS

Derivatization of hCG. Iodination of intact hCG was primarily confined to the α subunit. The degree of derivatization of hCG was determined by monitoring photolysis-dependent crosslinking of α and β subunits to $\alpha\beta$ dimer. The radioactivity of the $\alpha\beta$ dimer band was approximately 2% that of the total when the hormone was not photolyzed (Fig. 1; Table 1). This low radioactivity probably presents a minor population of hormone molecules that failed to dissociate even in the detergent solution. Beyond the background radioactivity of the $\alpha\beta$ dimer band, the conversion of monomers to dimers was dependent on both photolysis and the reagent concentration. Because the probability of forming a crosslink is <1 for a phenylnitrene generated by the photolysis of the phenylazide group (10), the concentration of the starting ABGG-^{125}I-hCG will be higher than that of the crosslinked dimer product. We assume that nearly one-half of the hCG was derivatized at the reagent concentration of 100 μM. In subsequent experiments, hCG was derivatized at that concentration. The hormone is known to contain six lysine residues in the α subunit and five in the β (11). The above data suggest that not all of the free amino groups were modified by the reagent.

Binding Activity of ABGG-^{125}I-hCG. The binding activity of the modified hormone was tested for its specificity, number

Table 1.   Crosslinking of α and β subunits of hCG

| | Distribution (%) of radioactivity at various reagent concentrations | | | |
	50 μM	100 μM	150 μM	150 μM*
$\alpha\beta$ dimer	22.8	37.3	67.2	2.2
α monomer	77.2	62.7	32.8	97.8

The hormone was derivatized, iodinated, fractionated, photolyzed for crosslinking, and analyzed by gel electrophoresis as described in the legend to Fig. 1.
* No photolysis.

Table 2.   Competitive inhibition of ABGG-^{125}I-hCG binding

| | Bound hCG, | |
Competitor	cpm	%
None	67,200	100
hCG	4,700	7.2
Lutropin	6,300	9.4
Follitropin	64,900	96
Prolactin	67,000	99
Insulin	68,200	101
Bovine serum albumin	66,500	99

Granulosa cells were incubated with the derivatized ^{125}I-hCG in the presence or absence of competitors for 30 min at 25°. The cells were rinsed twice and the bound radioactivity measured.

of binding sites, and affinity. ABGG-^{125}I-hCG bound to granulosa cells and the specific binding was calculated by subtracting nonspecific binding (the binding in the presence of 1000-fold higher concentration of untreated hCG) from the total binding. Nonspecific binding was less than 15% of the total binding above 100 pM and less than 2% below 100 pM. The specific binding of ABGG-^{125}I-hCG to granulosa cells was completely blocked to the basal level by excess untreated hCG or lutropin but not by follitropin, prolactin, insulin, or bovine serum albumin (Table 2). The number of binding sites on granulosa cells was equal for ABGG-^{125}I-hCG and ^{125}I-hCG, but the equilibrium association constants differed slightly, 2.98 × 10^9 M^{-1} for ABGG-^{125}I-hCG and 5.1 × 10^9 M^{-1} for ^{125}I-hCG (Fig. 2). Both values, however, were of the same order as K_a values reported for ^{125}I-hCG binding to porcine granulosa cells (12). Because one-third to one-half of the hCG was derivatized, these results suggest that ABGG-^{125}I-hCG bound specifically to the receptors as did untreated hCG.

Crosslinking of ABGG-^{125}I-hCG to the Receptor. Granulosa cells incubated with ABGG-^{125}I-hCG for 30 min at 37°C were rinsed and photolyzed. Membranes were solubilized and electrophoresed as described above. The photolyzed sample revealed three new bands of lower electrophoretic mobility in
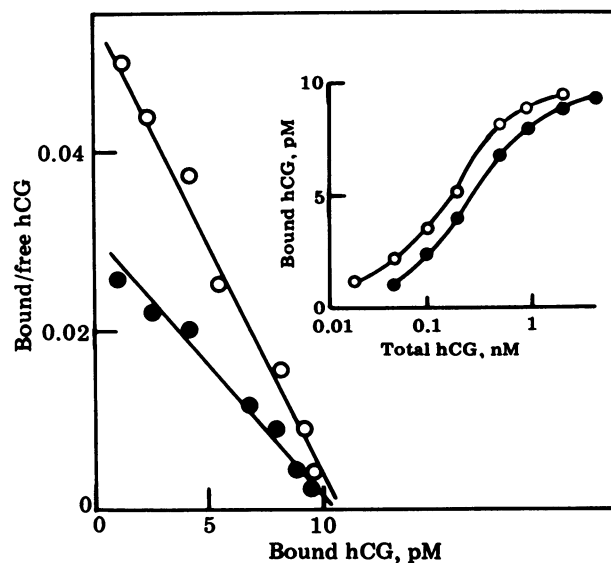

FIG. 2. Binding of ^{125}I-hCG and ABGG-^{125}I-hCG to granulosa cells. Granulosa cells were incubated with ^{125}I-hCG (O) or with ABGG-^{125}I-hCG (●) for 30 min at 37°C, rinsed twice, and assayed for bound radioactivity. Specific binding was estimated by subtracting nonspecific binding (hormone binding in the presence of 1000-fold higher concentrations of native hCG) from total binding. Nonspecific binding ranged from 15% to 2% of total binding.

Biochemistry: Ji and Ji

Proc. Natl. Acad. Sci. USA 77 (1980)   7169

addition to the α, β, and $\alpha\beta$ bands (Fig. 3). In the nonphotolyzed sample, there was a trace of the bands but their intensity was considerably less. When the cells were incubated with ABGG–^{125}I-hCG in the presence of excess untreated hCG and photolyzed, the three bands did not appear. Derivatized ^{125}I-hCG free in solution crosslinked to $\alpha\beta$ dimer upon photolysis but failed to yield higher molecular weight bands equivalent to the three bands; careful examination of the autoradiograph revealed traces of slower moving components in the photolyzed hormone, but their positions did not correspond to those of the three bands and their intensities were considerably less.

In view of these results, we conclude that the three new bands in (Fig. 3, lane E) are the product of photocrosslinking and contain the hormone (either the whole molecule or its subunits) in addition to some components other than the hormone. The simplest assignment for the identity of the unknown component is a membrane molecule because its formation was dependent on binding of the hormone to the membrane receptor. The present data, however, do not reveal the composition and precise identity of the photoaffinity-labeled components. Therefore, we conducted the following additional experiments to resolve some of the difficulties. When cells were incubated with varying concentrations of ABGG–^{125}I-hCG, rinsed, photolyzed, solubilized, and electrophoresed, the radioactivities of the three bands of crosslinked complexes turned out to be proportional to the quantity of the hormone specifically bound to the cells. The ratio of the radioactivities of the three bands was constant and their equilibrium association constant was in the order of 1×10^9 M^{-1}. Furthermore, the appearance of the three bands was independent of the incubation time of the cells with the hormone (from 30 min to 4 hr) and of the photolysis time period (milliseconds to several minutes), suggesting that the three bands represent stable complexes and do not involve nonspecific random collisional membrane components (8).
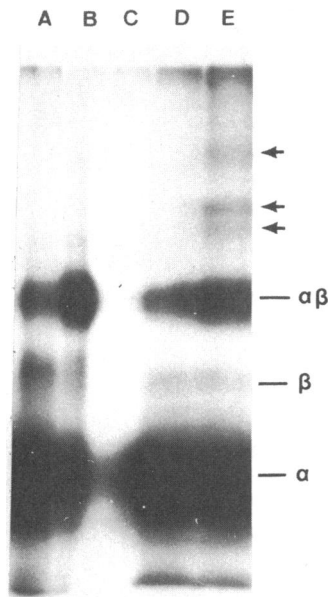
Although the crosslinking studies alone are not of themselves very convincing, when those data are combined with the equilibrium association constant as well as the result of the concentration and time-dependent studies, it seems reasonable to conclude that the photoaffinity labeling is specific and restricted to membrane components related to the hormone receptor. Besides, previous studies have demonstrated that macromolecular photoaffinity labeling is restricted to specific receptor complexes (13, 14). It is unlikely that potential microcontaminants of the hCG preparation were involved in the formation of the new bands because the most likely contaminant, follitropin (15), and also other hormones failed to block formation of them (data not shown).

The positions of the three bands on the gel correspond to molecular weights of $96,000 \pm 6,700$, $66,000 \pm 4,600$, and $63,000 \pm 4,400$, whereas the molecular weights of α, β, and $\alpha\beta$ are 23,000, 36,000, and 49,000, respectively. These molecular weights are tentative and probably larger than the true values (for example, 18,000, 28,000, and 46,000 for α, β and $\alpha\beta$, respectively) because of the abnormally low electrophoretic mobility caused by the high carbohydrate content of both subunits (>15% of their mass). The molecular weight of the photoaffinity-labeled components is probably less than 85,000 which is within the range 80,000–90,000 suggested by results of affinity column chromatography (2, 3).

DISCUSSION

We have demonstrated that certain membrane components of granulosa cells can be photoaffinity-labeled with ABGG–^{125}I-hCG and that these labeled complexes are resolved into three distinct bands by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The three bands cannot possibly be products of the degradation of hormone–receptor complexes as suggested (16) because they were formed not only by granulosa cells but also by isolated membranes. Our data do not demonstrate the composition or identity of the photoaffinity-labeled membrane components but merely suggest that they are specific and are related to the hormone receptor. It is yet to be determined whether or not they bind the hormone.

It has been suggested that the hormone receptor has a molecular weight of 194,200 and is composed of two subunits of molecular weight 90,000 (2). Others have indicated that the receptor has an 80,000-dalton hormone-binding component, and another 50,000-dalton component is persistently present in the receptors purified by affinity column chromatography (3). When this component was eluted from sodium dodecyl sulfate/polyacrylamide gels, it failed to bind to the hormone and was considered to be a contaminant. We do not know whether or not any of these components are related to the photoaffinity-labeled membrane components in our study.

It is of interest to know how many components are associated with the lutropin receptor. The presence of multiple components has been reported for the insulin receptor (17, 18) and the acetylcholine receptor (19). On the other hand, a single component receptor has been reported for glucagon, thrombin, and epidermal growth factor (13, 20).

The success of the crosslinking depends on the length, hydrophilicity of the reagents (see ref. 6 for a review), and the conditions under which they are coupled to hormones. Polypeptide hormones are known to be extremely susceptible to denaturation even under physiological conditions. Clearly, it is advantageous to convert them to their derivatives quickly—for example, 30 min in aqueous buffers in contrast to the earlier practice involving 24 hr in organic solvents (6).

FIG. 3. Crosslinking of ABGG–^{125}I-hCG to receptors on granulosa cells. The cells were incubated with ^{125}I-hCG for 30 min at 37°C in medium 199 containing 20% porcine serum, rinsed twice, and photolyzed. The resulting slab gel was dried and autoradiographed. Lanes: A, untreated ABGG–^{125}I-hCG; B, same as A but photolyzed; C, cells were incubated with the ABGG–^{125}I-hCG in the presence of excess untreated hCG and photolyzed after rinsing; D, cells were treated with ABGG–^{125}I-hCG but not photolyzed; E, same as D but photolyzed. Arrows indicate receptor–hormone crosslinked components.

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