Subunits of Luteinizing Hormone-Human Chorionic Gonadotropin Receptor from Bovine Corpora Lutea†

Brij B. Saxena,* Bosukonda Dattatreyamurty,‡ Hirotaka Ota,§ Ventzeslav Milkov,‖ and Premila Rathnam

Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, The New York Hospital-Cornell University Medical College, New York, New York 10021

Received March 6, 1986; Revised Manuscript Received August 20, 1986

ABSTRACT: A batch of 24 mg of luteinizing hormone-human chorionic gonadotropin (LH-hCG) receptor was isolated from bovine corpora lutea. The LH-hCG receptor showed specific binding with hCG. The receptor–hCG complex activated the regulatory N_s protein isolated from rabbit liver, which in turn stimulated adenylate cyclase to convert ATP into cAMP in vitro, attesting to the biological activity of the purified LH-hCG receptor. The LH-hCG receptor was treated with 2% sodium dodecyl sulfate (SDS) to prepare the molecular weight (M_r) 280K dimer and with 50 mM dithiothreitol (DTT) to prepare the M_r 120K monomer and subunits of M_r 85K and 38K. Oligomers of various molecular weights were recovered from gel filtration columns due to the reassociation of disulfide bonds between monomers and subunits. Hence, the receptor monomer was also dissociated into subunits of M_r 85K and 38K by reduction of –S–S– bonds with 50 mM DTT in 2% SDS and alkylation of sulfhydryl groups in the presence of 100 mM *N*-ethylmaleimide. The subunits were separated by gel filtration through columns of Ultrogel AcA-44 and Sephadex G-75. The yields of the purified alkylated subunits of M_r 85K and 38K were 1.8 and 1.5 mg, respectively. Each subunit migrated as a single entity in SDS–polyacrylamide gel electrophoresis. The monomer of the receptor of M_r 120K showed specific binding with ^{125}I-hCG, sugesting it to be the minimum molecular weight functional unit of the receptor. The M_r 85K and 38K subunits bound ^{125}I-hCG, which could not be displaced with unlabeled hCG. However, alkylated subunits of M_r 85K and 38K did not bind ^{125}I-hCG. These results may suggest an involvement of covalent linkage of disulfide bonds in the binding of ^{125}I-hCG to the receptor. The binding to ^{125}I-hCG of the unalkylated subunits represents partial reassociation of the subunits, since the alkylated subunits (which do not reassociate) do not bind ^{125}I-hCG. The monomer of M_r 120K of the receptor displaced 80% of the binding of the ^{125}I-receptor to the antibody raised against the LH-hCG receptor in rabbits, whereas the subunits of M_r 85K and 38K displace only 20% and 10%, respectively, also suggesting the immunological integrity of M_r 120K as the functional monomer, whereas the subunits of the LH-hCG receptor appeared immunologically nonidentical, substantiating their nonidentity suggested by gel filtration and SDS gel electrophoretic studies.

Receptors for polypeptide hormones are among the most sensitive and specific regulators of biological function in nature. Receptors in the cell membrane selectively bind to their specific hormones to form a hormone–receptor complex, which transmits the hormone stimulus into the cell to activate the adenylate cyclase system. The β-adrenergic receptor complex has been shown to stimulate adenylate cyclase by first activating N_s (also known as the G/F or G protein), the GTP binding stimulatory protein (Pederson & Ross, 1982). The activation of this regulatory N_s protein depends upon its phosphorylation from the hydrolysis of GTP to GDP. In this study, we have shown evidence for the activation of GTP-dependent N_s by the human chorionic gonadotropin (hCG)[1]–receptor complex, which in turn stimulates adenylate cyclase to catalyze in vitro the conversion of labeled ATP to cAMP (Saxena et al., 1985).

The LH-hCG receptor of rat ovary has been suggested to have components of M_r 50K (Saxena & Rathnam, 1976) or of M_r 65K (Metsikko & Rajaniemi, 1980) and from rat Leydig cells to have two identical subunits of M_r 90K (Catt & Dufau, 1978). Other studies employing photoaffinity labeling (Ji & Ji, 1980) and chemical cross-linking experiments (Bauknecht & Sievers, 1981) have shown the presence of multiple components of M_r 25K–96K in the LH-hCG receptor of porcine granulosa cells.

The LH-hCG receptor isolated from bovine corpora lutea appears to exist as an aggregate of apparent M_r of 5.9 million, which after treatment with sodium dodecyl sulfate (SDS) disaggregates into the dimeric form of M_r 280K. Each dimer is composed of two identical monomers of approximately M_r 120K–140K, and the monomer can be dissociated into two disulfide-linked, nonidentical subunits of M_r 85K and 38K by treatment with 2% SDS and 50 mM DTT (Dattatreyamurty et al., 1983). In this study, we have described the dissociation of the LH-hCG receptor subunits by reduction with 50 mM DTT in the presence of 2% SDS alone as well as after alkylation with NEM, and the separation of subunits by gel filtration. Further, the subunits of the receptor have shown

† This research was supported in part by grants from the Andrew Mellon Foundation, The Robert Wood Johnson, Jr., Charitable Trust, and the National Institutes of Health (Grant ROI CA 13908).

‡ Present address: Department of Biochemistry, Albany Medical College of Union University, Albany, NY 12208.

§ Present address: 344, 2-Chome, Itsutsubashi, Sendai 980, Japan.

‖ Present address: Institute of Roentgenology and Radiobiology, Medical Academy, Radioimmunoassay Laboratory, T43T Sofia, Bulgaria.

[1] Abbreviations: LH, luteinizing hormone; hCG, human chorionic gonadotropin; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; M_r, relative molecular weight; BSA, bovine serum albumin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PEG, poly(ethylene glycol).

0006-2960/86/0425-7943$01.50/0

immunological nonidentity in a radioimmunoassay of the LH-hCG receptor.

Materials and Methods

Materials

Highly purified hCG was a gift from Dr. R. E. Canfield (College of Physicians and Surgeons, Columbia University, New York, NY) and from Dr. O. P. Bahl (State University of New York, Buffalo, NY). Sephadex G-75 and high molecular weight calibration kits were obtained from Pharmacia. Ultrogel AcA-44 was purchased from LKB Instruments. Triton X-100 was obtained from J. T. Baker Chemical Co. Diaflo UM-10 membranes were obtained from Amicon Corp. Bovine serum albumin (BSA) and γ-globulin were purchased from Sigma. Sodium dodecyl sulfate (SDS) and *N*-ethylmaleimide (NEM) were obtained from Pierce Chemicals. Acrylamide was purchased from Mallinckrodt Chemicals, and *N*,*N*′-methylenebis(acrylamide) was obtained from ICN Pharmaceuticals. Carrier-free $Na^{125}I$ was supplied by Amersham. DEAE-Sephacel was purchased from Pharmacia, Piscataway, NJ; [α-^{32}P]ATP was obtained from New England Nuclear, Boston, MA; and GTP was from Sigma, St. Louis, MO. Frozen rabbit livers were purchased from Pel-Freeze, Rigers, AK.

Methods

Preparation of LH-hCG Receptor from Bovine Corpora Lutea. The LH-hCG receptor was prepared as described (Dattatreyamurty et al., 1983).

Preparation of Adenylate Cyclase from Bovine Corpora Lutea. A 160-g sample of bovine corpora lutea was homogenized twice with equal volumes of 10 mM Tris-HCl buffer of pH 8 containing 1 mM EDTA, 0.1% mercaptoacetic acid, 3 mM $MgCl_2$, and 10% sucrose (designated hereafter as the "TEB buffer"). The homogenate was filtered through two layers of cheesecloth and centrifuged at 5000 rpm for 30 min. The supernatant was extracted with an equal volume of chilled petroleum ether for 1 h at 4 °C to remove neutral lipids. The mixture was centrifuged at 10000 rpm for 1 h. The aqueous layer was concentrated by ultrafiltration through a PM-30 membrane. The concentrate was centrifuged at 55000*g* for 1 h. The pellet containing the adenylate cyclase was suspended in TEB buffer and gel filtered on a Sepharose-6B column to separate the adenylate cyclase fraction from the LH-hCG receptor fraction (Khan et al., 1981).

Preparation of N_s Regulatory Protein. The N_s regulatory protein was purified from frozen rabbit liver by a modification of the procedure of Sternweis et al. (1981). A batch of 900 g of frozen rabbit livers was pulverized and thawed at 4 °C in 2 L of 10 mM EDTA and 0.1% β-mercaptoacetic acid (TEB) buffer of pH 8.0. The thawed pieces of liver were homogenized in an equal volume of approximately 6 L of the TEB buffer. The homogenate was centrifuged at 10620*g* for 30 min. The pellet was suspended in 2.5 L of TEB buffer and centrifuged for 1 h at 31000*g* at 4 °C. The pellet was resuspended in 2.5 L of TEB buffer containing 0.1% NaCl and 0.1% sodium cholate and again centrifuged for 1 h at 4 °C at 31000*g*. The resulting pellet was homogenized in a total volume of 0.5 L of 20 mM Tris-HCl buffer of pH 8 containing 1 mM EDTA and 1 mM dithiothreitol (DTT). The homogenate was extracted at 4 °C by stirring for 1 h in an equal volume of TEB buffer containing 2% sodium cholate. The extract was concentrated by ultrafiltration through an Amicon PM-30 membrane to a volume of 650 mL and centrifuged at 15000*g* for 1 h at 4 °C. The supernatant was mixed with the stabilizing buffer of pH 8.0, containing 0.1 M ATP, 1 M $MgCl_2$, and 1 M NaF in a ratio of 100:1, and reconcentrated to a final volume of 25 mL. Both the supernatant and the pellet contained adenylate cyclase activity and showed little specific binding to ^{125}I-hCG.

An aliquot of 25 mL of the concentrated supernatant was applied to a 1.5 × 30 cm column of DEAE-Sephacel, which was previously equilibrated with TEB–AMF buffer (TEB buffer containing 0.1 M ATP, 1 M $MgCl_2$, 1 M NaF, and 0.9% sodium cholate). The DEAE-Sephacel column was eluted by a linear gradient from 0 to 250 mM NaCl in TEB–AMF buffer containing 0.9% sodium cholate. Fractions of 10 mL were collected and analyzed for protein concentration. The fraction containing the N_s protein activity was further purified by chromatography on Ultrogel AcA-34, Sepharose-4B, DEAE-Sephacel, and Sephadex G-25. The fraction containing the G protein was stored at −80 °C.

Assay of Adenylate Cyclase Activity. The adenylate cyclase activity was measured in vitro as the conversion of [^{32}P]ATP to [^{32}P]cAMP by the N_s-mediated stimulation of adenylate cyclase. A 25-μg aliquot of the G-protein fraction in 50 μL of 20 mM Tris-HCl buffer, containing 1 mM DTT, 2% sodium cholate, 1 mM EDTA, 1 mM ATP, 10 mM $MgCl_2$, and 10 mM sodium fluoride, was added to the tubes assayed. An aliquot of 50 μL of [^{32}P]ATP containing 10 μg of protein equivalent to approximately 100000 cpm was used in the assay. An aliquot of 20 μg of protein of the adenylate cyclase was added to each tube. One hundred micrograms of protein equivalent to the LH-hCG receptor and 5 μg of hCG was used per tube. A 50-μL aliquot of a 150 mM solution of GTP-PNP and a 50-μL aliquot of a 100 unit/mL solution of a 0.25 M Tris-HCl buffer of pH 7.5 containing 5 mM $MgCl_2$, 20 mM creatine phosphate, and 1 mM cAMP were added to all tubes. A 100-μL aliquot of a 1 mg/mL solution of BSA was used per tube. All tubes contained GTP-PNP, creatine phosphokinase, creatine phosphate, Tris-HCl–EDTA buffer, BSA, and [^{32}P]ATP. A "blank tube" (tube 1) did not have adenylate cyclase. Another blank tube (tube 2) did not have G protein. Tube 3 did not have receptor or hCG. Tube 4 contained hCG but not the receptor. Tube 5 contained receptor but not the hCG. Tube 6 contained both receptor and hCG. All the reagents were added with the tubes on ice. The contents of the tubes were incubated for 5 min at 30 °C, extracted with alcohol (2:1 volume), and centrifuged for 30 min at 3600 rpm. Both the aqueous and alcohol layers were separately evaporated under nitrogen. Each residue was redissolved in 200 μL of 0.05 M, pH 7.5, Tris buffer containing 4 mM EDTA, obtained from the kit for cAMP from Amersham. To 100 μL of the above solution was added 100 μL of cAMP binding protein solution (from the Amersham kit for cAMP), and the tubes were incubated for 2 h at 4 °C. One hundred microliters of Dextran-coated charcoal was added with the tubes kept in ice and mixed, and the tubes were centrifuged at 5000 rpm for 20 min. Five milliliters of distilled water or scintillation cocktail was added to an aliquot of the supernatant, and the tubes were counted in a scintillation counter. The cAMP produced was extracted with alcohol (2:1). The tubes were centrifuged at 3400–3600 rpm for 30 min. The supernatants containing cAMP were transferred to fresh tubes and evaporated to dryness. The dry residue containing cAMP was suspended in 50 μL of Tris–EDTA buffer and analyzed for cAMP content according to the protocol of the commercial kit (Amersham). The results are expressed as percent B/B_0 vs. the concentrations of the unlabeled cAMP used as standard. From the standard curve, cAMP concentrations of the unknown tubes were derived directly correspondent to the re-

spective B/B_0 in terms of picomoles of cAMP per tube. The results are expressed as picomoles of cAMP per minute per milligram of protein.

Isolation of the Subunits of LH-hCG Receptor. (*A*) *Preparation of the LH-hCG Monomer and Reduction with 50 mM DTT.* Twenty-four milligrams of highly purified LH-hCG receptor was solubilized by sonication twice at 50 W for 5 s each time and treated with 2% SDS for 90 min at 37 °C under N_2 and was gel filtered on a column of Sepharose-6B at 4 °C (Figure 1). Fractions I and II from the Sepharose-6B column, containing a large molecular weight aggregate and the receptor dimer of M_r 280K, were pooled, lyophilized, and retreated as above with 2% SDS and reincubated for 90 min at 37 °C. The sample was then centrifuged at 2500 rpm, and the clear supernate was fractionated on a column of Ultrogel AcA-34 (Figure 2). Fractions V and VI from Figure 2 contained the M_r 85K and 35K subunits but in low yields. Fractions I, II, and III from this column, containing the higher molecular weight oligomer formed due to the reassociation of disulfide bonds (Figure 2), were made 50 mM in DTT and 2% in SDS and incubated for 12 min at 37 °C. The incubate was then gel filtered on another column of Ultrogel AcA-34 (Figure 3). Fractions III and IV contained the M_r 85K and 38K subunits. The subunits were not completely separated, possibly due to their reassociation. Hence, the 16 mg of LH-hCG receptor monomer pooled from Sepharose-6B and Ultrogel AcA-34 columns (Figures 1–3) was dissociated into subunits by reduction with 50 mM DTT and 2% SDS, which was followed by alkylation with NEM, prior to the separation of the subunits by gel filtration.

(*B*) *Dissociation of the LH-hCG Receptor Monomer into Subunits by Reduction with 50 mM DTT Followed by Alkylation with NEM.* The LH-hCG receptor monomer (16 mg) was dissolved in 0.05 M Tris-HCl buffer of pH 7.5 containing 2% SDS and 1 mM EDTA. The sample was sonicated at 50 W twice for 5 s each time, made 50 mM in DTT, and incubated for 12 min at 37 °C to reduce the disulfide bonds. The sample was further made 100 mM in NEM and incubated for 10 min at 37 °C in order to alkylate the free sulfhydryl groups as well as to quench excess DTT.

(*C*) *Separation of the Subunits by Chromatography on Ultrogel AcA-44 and Sephadex G-75 Columns.* The reduced and alkylated sample was centrifuged, and the supernate was gel filtered on a column of Ultrogel AcA-44. The fractions eluted from the column were pooled as indicated in Figure 4. Fraction III from the Ultrogel AcA-44 column containing a mixture of the 85K and 38K subunits was further purified by gel filtration through a column of Sephadex G-75 to separate the 85K and the 38K subunits (Figure 5).

Analytical Methods. (*A*) *Protein Determination.* The protein concentration of each fraction was measured in the presence of Triton X-100 according to the method of Lowry et al. (1951), with modification as described earlier (Dattatreyamurty et al., 1983). Prior to protein determination, the fractions containing DTT and NEM were dialyzed overnight, at 4 °C, against the 10 mM Tris-HCl buffer of pH 7.0. The concentrations of Triton X-100 present in these samples were determined by the method of Garewal (1973).

(*B*) *SDS–Polyacrylamide Gel Disc Electrophoresis.* The LH-hCG receptor and its monomer and subunits were analyzed by SDS–polyacrylamide gel disc electrophoresis according to the method of King and Laemmli (1971).

(*C*) *Hormone Binding Capacity of the Receptor and Its Monomer and Subunits.* The assay procedure was the same as described previously (Dattatreyamurty et al., 1983). Two hundred micrograms of protein equivalent of the LH-hCG receptor was incubated overnight with ^{125}I-hCG (50000 cpm), alone and with unlabeled hCG (100 μg) in 10 mM Tris-HCl radioreceptor assay buffer of pH 7.2 at 4 °C. The bound and free ^{125}I-hCG were separated by the addition of 0.5 mL of γ-globulin (final concentration 0.02%) and 1 mL of 20% PEG and centrifugation at 4000 rpm for 20 min. The supernate was aspirated, and the pellets containing the bound ^{125}I-hCG were counted in a γ counter (Micromedic) with 62% efficiency. The monomer and the subunits of the LH-hCG receptor (100–250 μg of protein) were incubated with 50000 cpm of ^{125}I-hCG (equivalent to approximately 1 μg), alone or in the presence of excess unlabeled hCG (up to 10 μg). The bound and free ^{125}I-hCG were separated by gel filtration on 1 × 30 cm columns of Ultrogel AcA-34 for the monomer and on 1 × 50 cm columns of Sephadex G-100 for the subunits. The columns were equilibrated and eluted with 10 mM Tris-HCl, containing 1 mM $MgCl_2$, 0.01% NaN_3, and 0.4% Triton X-100.

(*D*) *Radioimmunoassay of LH-hCG Receptor.* Antibodies to the LH-hCG receptor were raised in rabbits, as described previously (Dattatreyamurty et al., 1983; Khan et al., 1981). Highly purified LH-hCG receptor was iodinated by the Chloramine-T method (Hunter & Greenwood, 1962) with minor modifications, as described below. An aliquot of 50 μL of 0.5 M sodium phosphate buffer of pH 7.4 was added to 5 μg of highly purified LH-hCG receptor in 5 μL of 0.1 M sodium phosphate buffer of pH 7.4 containing 0.1% Triton X-100. To this were added 1 mCi of Na^{125}I and 20 μL of a solution of 1 mg of Chloramine-T/mL of phosphate buffer. The reaction was stopped after 5 s by the addition of 50 μL of sodium metabisulfite (2 mg/mL solution of the above buffer) followed by the addition of 0.5 mL of 0.1 M sodium phosphate buffer of pH 7.4 containing 0.4% Triton X-100. The labeled receptor was separated from free ^{125}I by gel filtration on a 1 × 50 cm column of Ultrogel AcA-34, eluted by 0.1 M sodium phosphate buffer containing 0.1% Triton X-100. A nonequilibrium radioimmunoassay with a double antibody method of separation of bound and free ^{125}I-receptors was established as described below.

The "RIA buffer" consisted of 125 mM phosphate buffer of pH 7.0 containing 0.02 M EDTA, 0.1% Triton X-100, and 0.1% human serum albumin. The total count (TC) tube contained only 100 μL of the ^{125}I-receptor solution, and the blank tube contained 100 μL of the ^{125}I-receptor and 150 μL of the RIA buffer. The antiserum to the receptor (50 μL, initial dilution of 1:2500) was incubated either with 50 μL of the RIA buffer for total binding (TB) or with known amounts of a 5 mg/mL solution of the LH-hCG receptor for total displacement (TD) for 24 h at 4 °C. At the end of the incubation, 100 μL of the ^{125}I-LH-hCG receptor solution (100000 cpm, specific activity 43–50 μCi/μg) was added to the tubes. The tubes were incubated further for 24 h at 4 °C. The antibody, viz., goat anti-rabbit γ-globulin (50 μL), was added to all TB and TD tubes and mixed. The tubes were centrifuged for 30 min at 3500 rpm. The supernatants were aspirated, and the radioactivity was counted in an Autogamma counter (Micromedic with 62% efficacy). The immunoreactivities of the native receptor, of its dimer, of its monomer, and of its subunits were determined quantitatively in the radioimmunoassay system.

Results and Discussion

Activation of Adenylate Cyclase by the hCG–Receptor Complex. The LH-hCG receptor used in the present studies showed specific binding to ^{125}I-hCG as well as stimulated

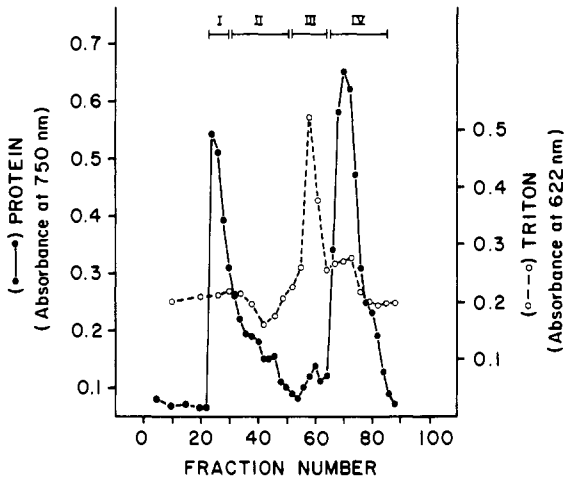

FIGURE 1: Gel permeation chromatography of LH-hCG receptor on a column (1.7 × 140 cm) of Sepharose-6B equilibrated and eluted with 0.1 M acetic acid containing 0.1% SDS, 1 mM $MgCl_2$, 0.5% Triton X-100, and 0.01% NaN_3. Receptor preparation (24 mg of protein) was dissolved in 10 mM Tris-HCl buffer containing 1 mM $MgCl_2$, 1 mM EDTA, and 0.5% Triton X-100 and treated with 2% SDS for 1.5 min at 100 °C before being applied to the column. Flow rate, 15 mL/h.

adenylate cyclase when bound to hCG, as described below. The activation of adenylate cyclase was measured in terms of the production of cAMP from ATP. The concentration of cAMP did not increase significantly from the basal level of 128 ± 34 pM min^{-1} (mg of G-protein)$^{-1}$ when the N_s protein was incubated with either receptor or hCG alone. However, when both the receptor and the hCG were incubated with the G protein, the concentration of cAMP increased 14.7-fold to 1893 ± 602 pM min^{-1} (mg of G-protein)$^{-1}$, indicating the stimulation of the adenylate cyclase by the hormone–receptor complex. Since the stimulation of the adenylate cyclase is also dependent upon an activated G protein, the results also indicate the activation of the G protein by GTP-PNP binding in the presence of the hormone–receptor complex.

It has been shown in other receptor systems that hormonal stimulation of adenylate cyclase occurs only in an unperturbed membrane (Ross & Gilman, 1980) or in "receptor vesicles" where the receptor is incorporated into a phospholipid bilayer (Pedersen & Ross, 1982). Solubilization of the plasma membrane or the addition of agents that disrupt membrane structure causes a loss of responsiveness to hormone. In an earlier paper from our laboratory (Haour & Saxena, 1974), it was shown that the emulsification of the purified receptor with the lipid fraction obtained during purification procedures enhanced the binding of the LH-hCG receptor. In the present studies, we used a receptor preparation which contained all the vital phospholipids.

Preparation of the LH-hCG Receptor Monomer and Dissociation of the Subunits with 50 mM DTT in 2% SDS. We have already shown evidence that the LH-hCG receptor exists as an aggregate of M_r 5.9 million, which after treatment with 2% SDS disaggregates to a dimer form of 280K. Whereas treatment of the 280K dimer with 1.5 mM DTT in 2% SDS causes dissociation into 120K monomeric forms, treatment with 50 mM DTT in 2% SDS dissociates both into two subunits of 85K and 38K (Dattatreyamurty et al., 1983).

Treatment of the LH Receptor with 2% SDS and Chromatography on the Sepharose-6B Column (Figure 1). Treatment of the purified LH-hCG receptor with 2% SDS caused up to 70% disaggregation of the receptor as shown by gel filtration on a Sepharose-6B column (Figure 1). Fractions I, II, III, and IV recovered from the Sepharose-6B column

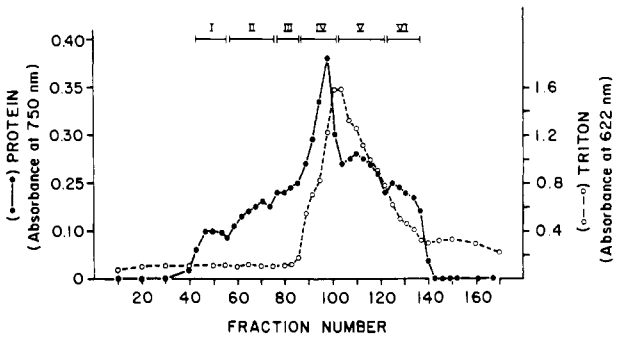

FIGURE 2: Gel permeation chromatography of LH-hCG receptor fractions I and II (from Sepharose-6B column, Figure 1) on a column (1.7 × 140 cm) of Ultrogel AcA-34 equilibrated and eluted with 10 mM Tris-HCl buffer of pH 7.2 containing 0.1% SDS, 1 mM $MgCl_2$, 1 mM EDTA, 0.4% Triton X-100, and 0.01% NaN_3. The pooled and lyophilized fractions, containing 18.6 mg of protein, were retreated with SDS as indicated in the legend for Figure 1 before being applied to the column. Flow rate, 15 mL/h.

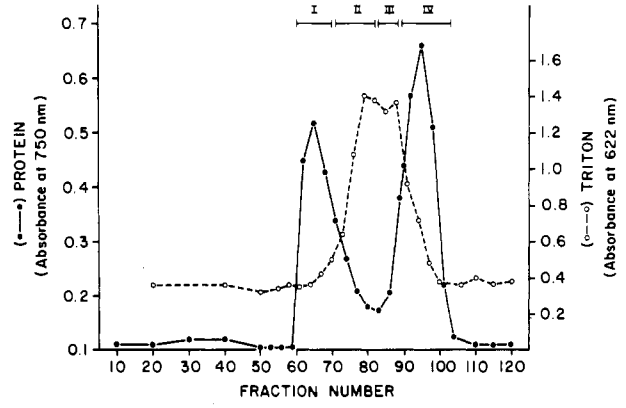

FIGURE 3: Gel permeation chromatography of LH-hCG receptor fractions (from Ultrogel AcA-34 column, Figure 2) on a column (1.7 × 140 cm) of Ultrogel AcA-34. Receptor fractions I, II, and III (Figure 2) were pooled and lyophilized. The pool containing 6.1 mg of protein was treated with 50 mM DTT in 2% SDS for 12 min at 37 °C before being applied to the column. Column elution buffer: 10 mM Tris-HCl buffer, pH 7.2, containing 0.1% SDS, 1 mM $MgCl_2$, 1 mM EDTA, 0.4% Triton X-100, and 0.01% NaN_3. Flow rate, 15 mL/h.

contained the undissociated receptor, the 280K dimer, the 120K–140K monomer, and the subunits of the receptor, with total protein contents of 6.1, 12.5, 3.1, and 2.9 mg, respectively.

Retreatment of fractions I and II of the Sepharose-6B column with 2% SDS resulted in a further substantial disaggregation as indicated by the increase in the M_r 280K dimer of the receptor obtained in fraction II obtained after gel filtration on a column of Ultrogel AcA-34. Fractions I and III, respectively, contained the 5.9 million aggregate and a 180K oligomer. Fractions, I, II, and III contained 1.9, 3.2, and 1.0 mg of total protein, respectively. The monomer of M_r 120K (fraction IV) and subunits of the receptor of M_r 85K (fraction V) and 38K (fraction VI) were also recovered from the Ultrogel AcA-34 column and contained 7.8, 0.7, and 3.3 mg of protein, respectively.

Reduction with 50 mM DTT and Chromatography on the Ultrogel AcA-34 Column. Fractions I, II, and III from the Ultrogel AcA-34 column (Figure 2), containing approximately 6.1 mg of protein, were pooled and reduced with 50 mM DTT. The dissociated components of the LH-hCG receptor were separated by gel filtration on a column of Ultrogel AcA-34, as shown in Figure 3. Fractions were pooled on the basis of electrophoretic mobility in SDS-polyacrylamide disc gel electrophoresis. Fractions I and II contained 180K and 120K

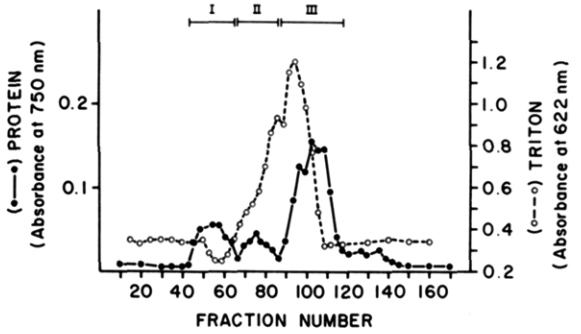

FIGURE 4: Gel permeation chromatography of the reduced and alkylated (120K–180K) LH-hCG receptor monomer fraction on a 2 × 88 column of Ultrogel AcA-44. Column elution buffer: 10 mM Tris-HCl buffer of pH 7.2 containing 0.1% SDS, 2 mM DTT, 1 mM $MgCl_2$, 0.4% Triton X-100, and 0.01% NaN_3. Flow rate, 14 mL/h.

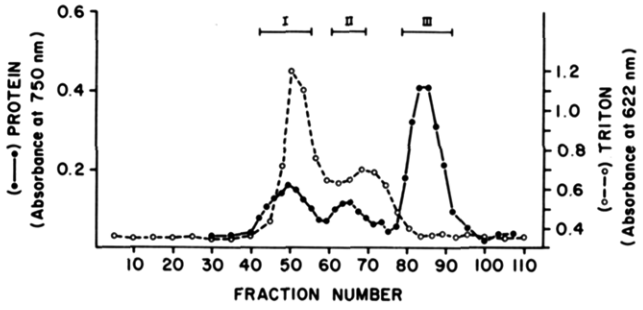

FIGURE 5: Separation of reduced and alkylated subunits of M_r 85K and 38K on a column (2.5 × 64 cm) of Sephadex G-75. The column was equilibrated and eluted with 10 mM Tris-HCl buffer of pH 7.2 containing 2 mM $MgCl_2$, 1 mM EDTA, 0.4% Triton X-100, and 0.01% NaN_3.

oligomers. Fractions III and IV contained predominantly the subunits of 85K and 38K, respectively. Fractions I, II, III, and IV contained 2.5, 1.4, 0.9, and 0.5 mg of protein, respectively. The treatment with 50 mM DTT further dissociated the LH-hCG receptor monomer into the 85K and 38K subunits, indicating the disulfide linkage between the subunits.

However, disulfide interchanges and reassociation of the subunits of the receptor occurred after reduction with DTT as indicated by the appearance of oligomers, for example, the M_r 180K oligomer, which caused incomplete separation and poor yield of the subunits. Hence, the reduction with 50 mM DTT was followed by alkylation with NEM to prevent any reassociation of the subunits.

Reduction with 50 mM DTT and Alkylation of the LH-hCG Receptor Monomer with NEM, and Separation of the Subunits. All fractions containing the receptor oligomers of M_r 120K–180K (Figures 1–3) were pooled and reduced with 50 mM DTT. The solution was further treated with NEM to alkylate the subunits and gel filtered on an Ultrogel AcA-44 column, as shown in Figure 4. The elution profile shows 1.4 mg of M_r 180K monomer in fraction I, 1.8 mg of M_r 120K monomer in fraction II, and 4.1 mg of a mixture of the subunits of M_r 85K and 38K in fraction III. A small but significant quantity of the receptor oligomer of M_r 180K was eluted from the Ultrogel AcA-44 column in fraction I. The M_r 180K species is suggested to be a dimer of the 85K subunit, due to incomplete alkylation of the 85K subunit. On retreatment with DTT and on realkylation, the 180K species yielded a single species of the M_r 85K subunit (unpublished observations).

Separation of the Alkylated Subunits by Gel Filtration on a Sephadex G-75 Column. Fraction III containing a mixture of the subunits of 85K and 38K of the LH-hCG receptor was gel filtered on a column of Sephadex G-75, as shown in Figure 5. The subunits of M_r 85K and 38K were separated in fractions I and II, with a yield of 1.8 and 1.5 mg of protein in each. Fraction III contained only buffer salts and low molecular weight inert material.

SDS–Polyacrylamide Gel Disc Electrophoresis. Each of the isolated subunits of the receptor migrated as a major single band in SDS–polyacrylamide gel disc electrophoresis, with molecular weight estimates of approximately M_r 85K and 38K, respectively, indicating a high degree of purity and electrophoretic homogeneity (Figure 6). Some of the minor components seen in the SDS gel electrophoresis may be caused by spontaneous disulfide interchanges. Since the subunits are recovered in definite yields and not in negligible quantities, they have to be derived from the major 120K component of the receptor only.

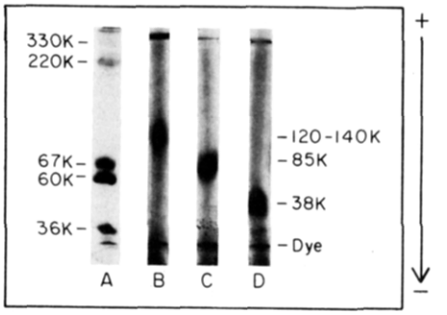

FIGURE 6: SDS–polyacrylamide disc gel electrophoresis of LH-hCG receptor monomer and subunits. Lane A, marker proteins: (1) thyroglobulin (330K); (2) ferritin (220K); (3) bovine serum albumin (67K); (4) catalase (60K); (5) lactate dehydrogenase (36K). Lane B, LH-hCG receptor monomer of M_r 120K–140K. Lane C, LH-hCG receptor subunit of M_r 85K. Lane D, LH-hCG receptor subunit of M_r 38K. Marker proteins were dissolved in 10 mM Tris-HCl buffer of pH 8.0 containing 1 mM EDTA and reduced by incubating for 15 min at 60 °C in the presence of 2% SDS and 1% mercaptoethanol. Samples dissolved in Tris-HCl buffer containing 0.4% Triton X-100 were heated in boiling water at 100 °C for 1.5 min in the presence of 2% SDS. Electrophoresis was performed in 7.5% acrylamide gels (pH 8.8), using 0.025 M Tris–glycine buffer, pH 8.3, containing 0.1% SDS. The stacking gels were prepared in 3% polyacrylamide at pH 8.0. Gels were stained with Coomassie Blue.

Hormone Binding Capacity of the Oligomers and Subunits of the LH-hCG Receptor. The monomer of the receptor of M_r 120K as well as the subunits of the receptor (85K and 38K) bound ^{125}I-hCG and eluted as hormone–receptor complexes from Ultrogel AcA-34 and Sephadex G-100 columns. In the presence of up to 5 μg of excess unlabeled hCG, there was a 42% inhibition of the binding of ^{125}I-hCG to the monomer of M_r 120K (Figure 7A). It is interesting to note, however, the SDS- and DTT-treated subunits bound labeled hCG; however, the bound hCG could not be displaced with excess unlabeled hCG (Figure 7 A,B). The fact that the subunits do bind ^{125}I-hCG indicates that they are derived from the LH-hCG receptor. Alkylation of the receptor subunits prevented their reassociation and resulted in complete loss of hormone binding capacity. The binding to ^{125}I-hCG of the unalkylated subunits also represents partial reassociation, since the alkylated subunits (where there is no reassociation of the disulfide bonds) do not bind ^{125}I-hCG. These observations suggest that disulfide groups may also be involved in the binding of LH or hCG to the receptor, similar to that of the insulin receptor interaction (Clark & Harrison, 1983).

Radioimmunoassay of LH-hCG Receptor. The purified LH-hCG receptor was labeled with ^{125}I to a specific activity of 43–54 μCi/μg. Antibody against the receptor, at an initial dilution of 1:2500, specifically bound up to 10% of the receptor

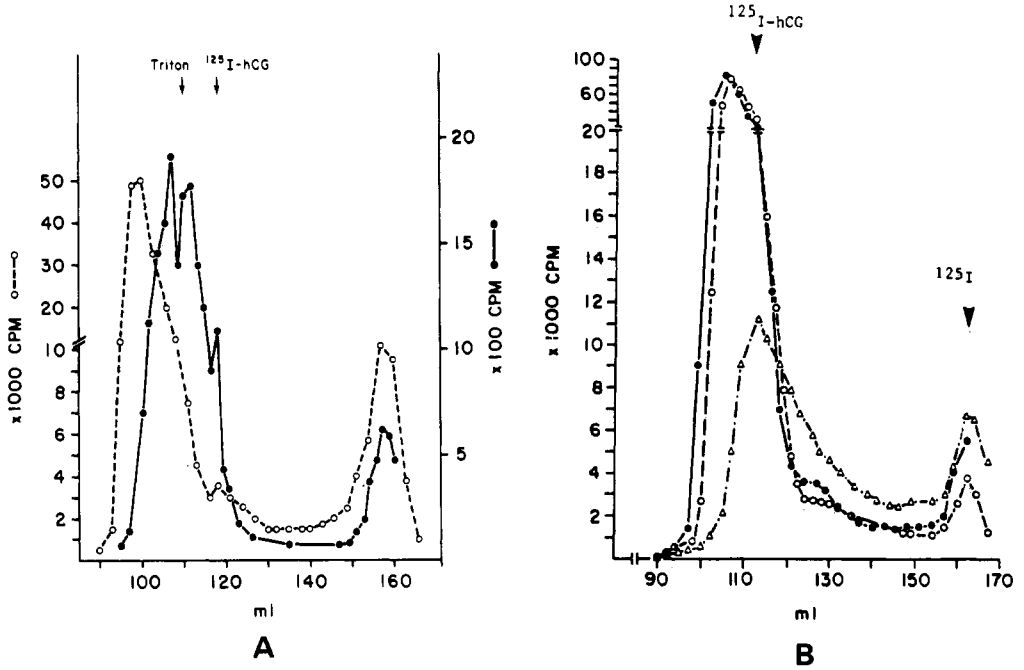

FIGURE 7: Binding of ^{125}I-hCG to LH-hCG receptor monomer (M_r 120000) and subunits (M_r 85000 and 38000). Reduced receptor monomer/subunits (see Methods) were preincubated with ^{125}I-hCG alone or with excess unlabeled hCG for 16 h at 4 °C, as described previously (Dattatreyamurty et al., 1983). Separation of free and receptor (monomer/subunit) bound ^{125}I-hCG was accomplished by gel permeation chromatography. (A) Gel permeation chromatography on Ultrogel AcA-34 column (1.3 × 135 cm); receptor monomer was incubated with ^{125}I-hCG alone (○) and in the presence of 5 μg of unlabeled hCG (×); receptor subunit (M_r 38000) was incubated with ^{125}I-hCG alone (●). (B) Gel permeation chromatography on Sephadex G-100 column (1.3 × 140 cm); ^{125}I-hCG alone (△); receptor subunit (M_r 85000) was incubated with ^{125}I-hCG (●) and in the presence of 10 μg of unlabeled hCG (○). Columns were equilibrated and eluted with 10 mM Tris-HCl buffer of pH 7.2 containing 1 mM $MgCl_2$, 0.01% NaN_3, and 0.4% Triton X-100. Arrows indicate peak elution volumes (V_e) of Triton X-100, ^{125}I-hCG, and ^{125}I.

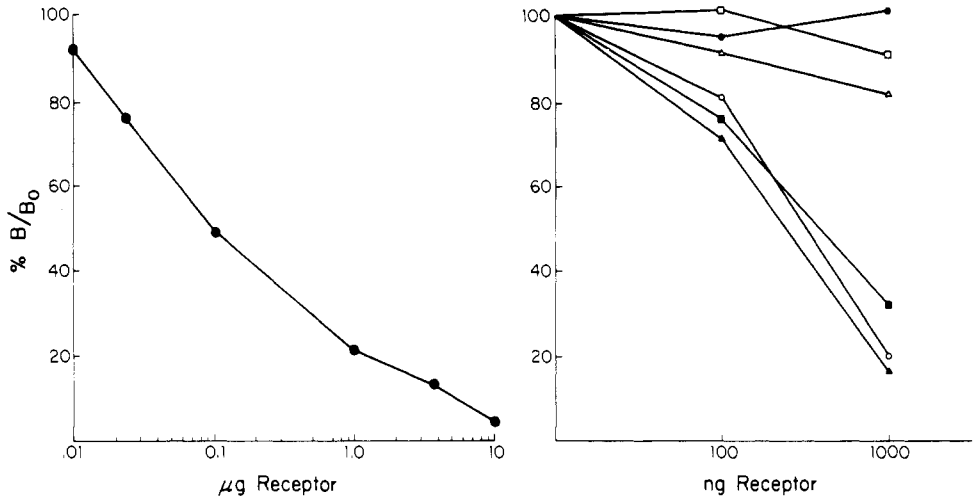

FIGURE 8: (A) Standard curve for the radioimmunoassay of LH-hCG receptor. Assay was carried out by using rabbit antibody to intact LH-hCG receptor (initial dilution 1:2500) and ^{125}I-LH-hCG receptor (approximately 100000 cpm) in the presence of increasing concentrations of purified LH-hCG receptor. A second antibody (goat anti-rabbit γ-globulin) was used for the separation of free and antibody-bound ^{125}I-LH-hCG receptor. The ^{125}I-LH-hCG receptor bound was plotted as a percentage of maximum total binding observed in the absence of unlabeled receptor. The specific activity of ^{125}I-LH-hCG receptor was 43–54 μCi/μg. (B) Competitive displacement of ^{125}I-LH-hCG receptor bound to anti-intact LH-hCG receptor antibody by LH-hCG receptor dimer (M_r 280K) (▲), monomer (M_r 120K) (○), preincubated receptor monomer and subunits (■), subunit (M_r 85K) (△), subunit (M_r 38K) (□), and preincubated receptor subunits (●). Radioimmunoassays were carried out as described under Methods, in the presence of indicated concentrations of pure receptor dimer/monomer/subunits. The ^{125}I-LH-hCG receptor bound was plotted as a percentage of the maximum total binding observed in the absence of any added receptor fractions.

in the presence of 0.4% Triton X-100. The low binding of the labeled receptor with the antibody was perhaps due to the presence of the detergent in the incubation mixture as was also noted in the case of the insulin receptor (Harrison et al., 1979; Endo et al., 1982). The binding of the ^{125}I-receptor to antibody was progressively inhibited by increasing amounts of the unlabeled receptor in a dose-dependent manner to yield a standard curve as shown in Figure 8A. The standard curve showed a sensitivity in the range of 10–20 ng or approximately 25–50 fM receptor.

As shown in Figure 8B, the 280K dimer, the receptor monomer, and the M_r 85K and 38K subunits showed only a little inhibition at a concentration of 1 μg of protein. A mixture of the subunits alone did not restore immunoreactivity to the level of the receptor dimer or monomer of M_r 280K and 120K. However, the subunits added to the monomer of M_r 120K

showed up to 70% inhibition of the binding of ^{125}I-hCG receptor to its antibody. These observations suggest that the monomer of M_r 120K–140K may represent the minimum molecular weight receptor unit, which is capable of specifically binding hCG. These results also attest to the immunological nonidentity of the subunits of the receptor.

Catt and DuFau in 1978 suggested that the hCG receptor isolated from rat Leydig cells is a dimer of two subunits of identical molecular weight (M_r 90K) which were linked together by disulfide bonds. Furthermore, the presence of multiple components of the LH-hCG receptor in the range of M_r 83K–120K has been reported in porcine ovarian cell membranes (Ji & Ji, 1980; Ji et al., 1981). It is likely that the subunit of M_r 38K, obtained in our studies, is buried in the hydrophobic lipid bilayer and is released only in detergent-solubilized membrane. A discrepancy in the identification of the insulin receptor subunits between photoaffinity labeling experiments and a direct analysis of purified receptor has been shown previously (Siegel et al., 1981; Jacobs et al., 1979; Massague & Czech, 1982). The presence of smaller molecular weight components of the LH-hCG receptor with molecular weights of 96K, 74K, and 48K has been reported (Rapoport et al., 1983; Kellokumpu & Rajaniemi, 1985). Recently, Wimalasena et al. (1985) have purified porcine LH-hCG receptor to a specific binding capacity of 2300 pmol/mg of protein and demonstrated the presence of two components of different molecular weights, namely, M_r 68K and 45K, by SDS gel electrophoresis. Our observations with LH-hCG receptor are similar to those present in the assembly of the insulin receptor from human placenta and rat liver (Siegel et al., 1981; Jacobs et al., 1979; Massague & Czech, 1982), namely, that the native receptor may consist of two heavy chains and two light chains and their configuration is stabilized by disulfide bonds.

Ascoli and Segaloff (1985) have obtained similar results both in a clonal strain of cultured Leydig tumor cells and in primary cultures of porcine granulosa cells where both subunits of the receptor-bound hormone become cross-linked to a single cellular component of M_r 106 000 when analyzed in the absence of reducing agents, and of M_r 83 000 when analyzed in the presence of reducing agents. Their results also suggest that when the LH-hCG receptor is cleaved by collagenase at one or more sites, the peptides so generated are held together by disulfide bonds, confirming the disulfide bond linkage between the subunits. Segaloff et al. (1986) found similar results with a clonal line of murine tumor cells. On the other hand, Kusuda (1986) reports that the LH-hCG receptor from rat ovary consists of two identical subunits of M_r 69 000.

The results obtained by us show that the 85K subunit and the 38K subunit are not identical. However, evidence to substantiate whether the 85K subunit consists of two 38K subunits will have to await structural characterization in terms of partial amino acid sequences of the LH-hCG receptor subunits. In summary, the observations presented provide evidence that the LH-hCG–receptor complex can activate the regulatory N_s protein to stimulate adenylate cyclase. The M_r 120K monomer of the LH-hCG receptor represents the functional unit of the receptor and consists of subunits of M_r 85K and 38K. The subunits are disulfide linked and are nonidentical as suggested from difference in their elution volume (K_d), SDS gel disc electrophoresis, and immunological activity in the radioimmunoassay of the LH-hCG receptor.

Registry No. ATP, 56-65-5; cAMP, 60-92-4; adenylate cyclase, 9012-42-4.

References

Ascoli, M., & Segaloff, D. L. (1986) *J. Biol. Chem. 261*, 3807–3815.

Bauknecht, T., & Siebers, J. W. (1981) *Acta Endocrinol. (Copenhagen), Suppl. No. 240*, 122.

Catt, K. J., & Dufau, M. L. (1978) in *Receptors and Hormone Action III* (Burnbaumer, L., & O'Malley, B. W., Eds.) pp 291–399, Academic Press, New York.

Clark, S., & Harrison, L. C. (1983) *J. Biol. Chem. 258*, 11434–11437.

Dattatreyamurty, B., Rathnam, P., & Saxena, B. B. (1983) *J. Biol. Chem. 258*, 3140–3158.

Endo, K., Borges, M., & Ingbar, S. H. (1982) *J. Clin. Endocrinol. Metab. 55*, 566–576.

Froehner, S. C., Reiness, C. G., & Hall, Z. W. (1977) *J. Biol. Chem. 252*, 8589–8596.

Garewal, H. S. (1973) *Anal. Biochem. 54*, 319–324.

Haour, F., & Saxena, B. B. (1974) *J. Biol. Chem. 249*, 2195.

Harrison, L. C., Flier, J. S., Roth, J., Karlson, F. A., & Kahn, C. R. (1979) *J. Clin. Endocrinol. Metab. 48*, 59–65.

Holloway, P. M. (1973) *Anal. Biochem. 53*, 304–308.

Hunter, W. M., & Greenwood, F. C. (1972) *Nature (London) 194*, 495.

Jacobs, S., Hazum, E., Shechter, Y., & Cuatrecasas, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4918–4921.

Ji, I., & Ji, T. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7167–7170.

Ji, I., Yoo, Y., Kaltenbach, C., & Ji, T. H. (1981) *J. Biol. Chem. 256*, 10853–10858.

Kellokumpu, S., & Rajaniemi, H. (1985) *Endocrinology (Baltimore) 116*, 707–714.

Khan, F. S., Rathnam, P., & Saxena, B. B. (1981) *Biochem. J. 197*, 7–22.

King, J., & Laemmli, U. K. (1971) *J. Mol. Biol. 62*, 465–473.

Kushner, L. H., & Hubbard, W. C. (1954) *J. Phys. Chem. 58*, 1163–1169.

Kusuda, S. (1986) 68th Annual Meeting of the Endocrine Society, Anaheim, CA, Abstr. 613.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Massague, J., & Czech, M. P. (1982) *J. Biol. Chem. 257*, 6729–6738.

Merlie, J. P., Changeux, J. P., & Gross, F. (1978) *J. Biol. Chem. 253*, 2882–2891.

Metsikko, K., & Rajaniemi, M. (1980) *Biochem. Biophys. Res. Commun. 95*, 1730–1736.

Patrick, J., Boulter, J., & O'Brien, J. C. (1975) *Biochem. Biophys. Res. Commun. 64*, 219–225.

Pedersen, S. E., & Ross, E. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7228–7232.

Rapoport, B., Hazum, E., & Zor, U. (1984) *J. Biol. Chem. 259*, 4267–4271.

Ross, E. M., & Gilman, A. C. (1980) *Annu. Rev. Biochem. 49*, 533–564.

Saxena, B. B., & Rathnam, P. (1976) in *Cellular Mechanism Modulating Gonadal Action* (Singhal, R. L., & Thomas, J. A., Eds.) pp 259–324, University Park Press, Baltimore, MD.

Saxena, B. B., Dattatreyamurty, B., Ota, H., & Rathnam, P. (1984) Abstracts of the 7th International Congress of Endocrinology, Quebec, Canada.

Saxena, B. B., de la Cruz, M., Shinkawa, O., Milkov, V., & Rathnam, P. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 1797.
Segaloff, D. L., Kim, L. C., & Ascoli, M. (1986) 68th Annual Meeting of the Endocrine Society, Anaheim, CA, Abstr. 612.
Siegel, T. W., Ganguly, S., Jacobs, S., Rosen, O. M., & Rubin, C. S. (1981) *J. Biol. Chem. 256*, 9266-9273.
Sternweis, P. C., Northup, J. K., Smiegel, M. D., & Gilman, A. G. (1981) *J. Biol. Chem. 256*, 11517-11526.
Wimalasena, J., Moore, P., Wieber, J. P., Abel, J., Jr., & Chen, T. (1985) *J. Biol. Chem. 260*, 10689-10697.

Activation of *Bordetella pertussis* Adenylate Cyclase by the Carboxyl-Terminal Tryptic Fragment of Calmodulin

J. Wolff,*,‡ Dianne L. Newton,§ and Claude B. Klee§

National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases and National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Received June 2, 1986; Revised Manuscript Received August 21, 1986

ABSTRACT: Highly purified tryptic fragments of calmodulin were tested for their ability to stimulate adenylate cyclase activity of *Bordetella pertussis* spheroplast membranes and were compared to their activities on brain Ca^{2+}/calmodulin-dependent cyclic nucleotide phosphodiesterase. The C-terminal fragment, consisting of residues 78–148, was a full agonist for the cyclase with 0.1–0.15 the potency of calmodulin but did not stimulate phosphodiesterase. Fragments 1–77, 1–90, and 107–148 stimulated adenylate cyclase (and not phosphodiesterase) at low potency; this was not due to calmodulin contamination, but contamination by fragment 78–148 could not be excluded with certainty. An adduct of norchlorpromazine isothiocyanate and calmodulin showed full agonist activity for adenylate cyclase at 0.01–0.02 the potency of calmodulin. Stimulation of adenylate cyclase by a number of the fragments occurred in the absence of Ca^{2+}, but stimulator potency was enhanced 20–60-fold in its presence. The similarity of Ca^{2+} requirements of fragment 78–148 and calmodulin suggests that occupancy of the two C-terminal Ca^{2+} binding sites of calmodulin accounts for most of the Ca^{2+} enhancement of calmodulin stimulation of adenylate cyclase.

Many enzymes are activated by calmodulin (Klee & Vanaman, 1982), and it has often been assumed that the mode of this activation is based on a similar calmodulin binding domain in these otherwise different target proteins. However, a number of the characteristics of the binding interaction show wide variations from one protein to the next: (1) the dissociation constants for calmodulin, usually in the nanomolar range, are much higher for MAP and τ (Lee & Wolff, 1984), spectrin/fodrin (Davies & Klee, 1981; Sobue et al., 1980), and troponin I (Keller et al., 1982); (2) Ca^{2+} is an obligatory participant in the reaction of calmodulin with most enzymes but is not required for interaction with phosphorylase kinase (Cohen et al., 1978), histones (Khandelwal et al., 1980), troponin I (Olwin et al., 1982), a brush border protein (Glenney & Weber, 1980), protein P-57 (Andreasen et al., 1983), and the adenylate cyclases of *Bordetella pertussis* (Greenlee et al., 1982; Kilhoffer et al., 1983) and *Bacillus anthracis* (Leppla, 1985).

An alternative approach to an understanding of such differential effects has been with the use of calmodulin derivatives. On the one hand, chemical modifications have led to differential effects on a number of calmodulin-sensitive enzymes (Thiry et al., 1980). On the other hand, limited proteolytic cleavage by trypsin or thrombin has yielded fragments containing residues 1–77 and 78–148, 1–90, 1–106, and 107–148, retaining one, two, or three of the Ca^{2+} binding domains (Drabikowski et al., 1977). Some of these can still activate certain enzymes but have lost all ability to activate phosphodiesterase and myosin kinase (Drabikowski et al., 1982; Guerini et al., 1984; Kuznicki et al., 1981; Newton & Klee, 1984; Newton et al., 1984, 1985; Wall et al., 1981; Walsh et al., 1977). Because the fragments have much lower affinities for the target proteins than calmodulin, it is important that contamination by calmodulin be $\ll$0.1%. Earlier studies were ambiguous because low levels of contamination by intact calmodulin could not be ruled out. Several recent studies have succeeded in overcoming this objection by substantial refinements in purification and detection methods (Guerini et al., 1984; Newton et al., 1984, 1985). Using these criteria, cyclic nucleotide phosphodiesterase and myosin kinase can now be shown not to be stimulated by any of the proteolytic fragments in concentrations up to 1×10^{-5} M. This stringent requirement for the intact regulator thus made the activation of phosphodiesterase (PDE)[1] a reliable detection system for contamination by calmodulin, and we have used tryptic calmodulin fragments so tested to assess their ability to activate the adenylate cyclase of *B. pertussis*.

Bordetella pertussis organisms contain a very active extracellular adenylate cyclase that is largely confined to the

‡ National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases.
§ National Cancer Institute.

[1] Abbreviations: PDE, phosphodiesterase; AC, adenylate cyclase; CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $CAPP_1$–calmodulin, 1 to 1 covalent adduct of 2-chloro-10-(3-aminopropyl)phenothiazine isothiocyanate with calmodulin; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.