MOLECULAR PHARMACOLOGY, 20:694–703

Functional Alterations in Components of Pigeon Erythrocyte Adenylate Cyclase following Desensitization to Isoproterenol

THOMAS H. HUDSON AND GARY L. JOHNSON

Section of Physiological Chemistry, Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

Received March 2, 1981; Accepted July 6, 1981

SUMMARY

In comparison with controls, membranes isolated from pigeon erythrocytes exposed to isoproterenol exhibit decreased adenylate cyclase activity. Fluoride and guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) activations are lowered in desensitized membranes and in Lubrol PX extracts of membranes previously treated with isoproterenol and GMP to remove GDP bound to the guanine nucleotide regulatory protein (G-protein). The decreases are not due to changes in K_{act} for either GTPγS or isoproterenol. The affinity and number of [^{3}H]dihydroalprenolol binding sites in desensitized membranes are similar to those found in control membranes. No functional differences are found between G-proteins solubilized from control and desensitized pigeon erythrocyte membranes as determined in reconstitution experiments with cyc$^-$ membranes. This laboratory recently has described [*J. Biol. Chem.* **256**:1459–1465 (1981)] techniques to monitor conformational changes in pigeon erythrocyte G-protein mediated by guanine nucleotides and hormone-receptor interactions. These involve partial tryptic digestion and peptide mapping of the cholera toxin-labeled M_r = 42,000 subunit of the G-protein. This procedure, carried out on control and desensitized membranes, demonstrates (a) that isoproterenol has a diminished ability to alter the conformation of the G-protein in desensitized membranes, (b) that a lag in the conformational change induced by GTPγS in the presence or absence of isoproterenol is observed in desensitized preparations, and (c) that the fraction of G-proteins found in the GTPγS-specific conformation is significantly decreased in desensitized versus control membranes incubated with GTPγS with or without isoproterenol. These findings indicate that alterations occur during desensitization which affect coupling of hormone receptor to G-protein and the GDP exchange reaction of adenylate cyclase.

INTRODUCTION

Hormone-sensitive adenylate cyclase is composed of at least three distinct functional units: hormone receptor, catalytic adenylate cyclase, and a regulatory component [variously termed G[1] (1), G/F (2), and N (3)]. The G-protein, which functionally couples receptor-hormone interaction with the activation of adenylate cyclase, requires a guanine triphosphate for its action (4). The effects of fluoride on the system are also mediated by the G-protein (5).

Catecholamine-sensitive adenylate cyclase of the avian erythrocyte appears to have GDP tightly bound to the regulatory site of the G-protein (6). In the absence of hormone, guanine nucleotides have little effect on cyclase activity (7, 8). Incubation of turkey erythrocyte membranes with isoproterenol and GMP (9) releases the GDP bound at the regulatory site of the G-protein. After washing of the membranes, Gpp(NH)p or GTPγS is capable of activating cyclase in the absence of hormone (9). Pigeon erythrocyte membranes appear to behave similarly (10). Because of this exaggerated property of "tight" binding of GDP to the regulatory site, the avian erythrocyte has been a particularly useful system with which to study the hormone-induced heterotropic displacement of GDP from the receptor-G-protein complex (6, 9).

A decrease in the hormonal stimulation of adenylate cyclase by prolonged exposure to agonist has been documented in both intact cells (11–14) and membrane systems (15–17). Although the ill-defined mechanisms involved in desensitization vary between hormone-responsive systems, some involvement of the individual

This work was supported by National Institutes of Health grants GM26776 and AM 007817, the American Heart Association with funds contributed in part by the American Heart Association, Rhode Island affiliate, and the Juvenile Diabetes Foundation.

[1] The abbreviations used are: G-protein, guanine nucleotide-binding regulatory component of adenylate cyclase; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); cyc$^-$, adenylate cyclase activity-deficient S49 cell variant; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetracetic acid; DHA, dihydroalprenolol; SDS, sodium dodecyl sulfate.

0026-895X/81/030694-10$02.00/0

cyclase components has been demonstrated. Desensitization of frog erythrocytes by *beta*-adrenergic agonists results in an uncoupling of receptor from the G-protein and a loss of high-affinity agonist binding sites (18), with both phenomena following similar time courses. In other systems (19) a functional uncoupling of receptor from the G-protein/catalytic components following exposure to agonist accounts for an initial loss in hormone responsiveness which precedes the loss of high-affinity receptors.

Incubation of turkey erythrocytes with *beta*-adrenergic agonists results in the loss of isoproterenol-stimulated adenylate cyclase activity (14). However, there is no apparent alteration in *beta*-receptor number or affinity for ligands following desensitization (14), suggesting an alteration distal to hormone-receptor interaction. More recently, Pike and Lefkowitz (20) have demonstrated a decrease in isoproterenol-stimulated GTPase of membranes from desensitized turkey erythrocytes. This raises the possibility of a receptor-G-protein coupling alteration which also activates the "turn off" mechanism of the system.

We have chosen the pigeon erythrocyte to study the desensitization of hormone-stimulated adenylate cyclase for two reasons: (a) the tight binding of GDP to the G-protein, which facilitates the study of heterotropic GDP displacement of hormone, and (b) the ability to label specifically the M_r = 42,000 subunit of the G-protein using cholera toxin and [^{32}P]NAD$^+$.

Techniques developed in this laboratory (21), involving partial proteolytic digestion of the cholera toxin-labeled protein, define various conformations assumed by the G-protein in response to hormone-receptor interactions in the presence or absence of guanine triphosphate. These characteristics are utilized here to delineate changes in receptor-regulatory protein interactions which occur during desensitization of the pigeon erythrocyte adenylate cyclase system.

MATERIALS AND METHODS

Cell culture. S49 cyc$^-$ mouse lymphoma cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum as previously reported (22).

Desensitization. Washed pigeon erythrocytes were prepared from freshly drawn citrated blood. After washing twice in Puck's saline G, the cells were resuspended in 10 mM Tris-HCl (pH 7.4), 0.2% glucose, 157.5 mM NaCl, and 2.5 mM KCl. The cells were incubated in this medium with (desensitized cells) or without (control cells) 10 μM (±)isoproterenol for 4 hr at 37°. The cells were then washed twice in Puck's saline G by dilution and centrifugation and used in the membrane preparations.

Isolation and solubilization of plasma membranes. Membranes from cyc$^-$ S49 cells were prepared by a modification (23) of the method described by Ross *et al.* (24). Pigeon erythrocytes were lysed in the presence of DNase, and membranes were prepared as described previously for turkey erythrocytes (4). Membranes were frozen in dry ice/ethanol and stored at −80°.

Detergent extracts of pigeon erythrocyte membranes were prepared by washing the membranes three times in 2.0 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 1 mM 2-mercaptoethanol (solubilization buffer). The membranes were then resuspended (9 mg of membrane protein per milliliter) in the solubilization buffer, and 10% (w/v) Lubrol PX was added to a final concentration of 0.7%. After a 30-min incubation on ice the lubrol extracts were centrifuged for 1 hr at 100,000 × *g* and the supernatant was removed and saved for either adenylate cyclase assay or reconstitution with cyc$^-$ membranes.

Approximately 30% of total membrane protein was extracted from both control and desensitized membranes. Similar amounts of G-protein were extracted from control and desensitized membranes based on the quantitation of [^{32}P]ADP-ribosylated G-protein in membranes and Lubrol PX extracts. Quantitation was performed by SDS gel electrophoresis followed by autoradiography and densitometry.

Radioligand binding. Binding of [^{3}H]DHA to control and desensitized pigeon erythrocyte membranes was assayed at 30° with 10-min incubations. Incubation mixtures contained 200 μg of membrane protein and [^{3}H]-DHA at the indicated concentrations in 100 μl (final volume) of 10 mM Tris-HCl and 2 mM $MgCl_2$ (pH 7.4) (binding buffer). Specific binding was defined by including 10 μM alprenolol in the binding mixtures and was greater than 70% of total [^{3}H]DHA bound. Unbound ligand was removed by vacuum filtration (using GF/C filters presoaked in binding buffer) followed by three 5-ml washes of ice-cold binding buffer. Results are reported as specific binding.

Adenylate cyclase assay. Adenylate cyclase was assayed in a mixture containing 0.4 mM [^{32}P]ATP (20–25 cpm/pmole), 50 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 0.2 mM EGTA, 2 mM 2-mercaptoethanol, bovine serum albumin (0.1 mg/ml), 10 mM creatine phosphate, and creatine phosphokinase (10 units/ml) in a final volume of 100 μl. Effectors of adenylate cyclase included in the reaction mixture unless otherwise noted were as follows: 10 mM NaF, 10 μM l-isoproterenol, 100 μM GTP, or 100 μM GTPγS. The assay was initiated by adding 20–40 μg of membrane or Lubrol PX-extracted membrane protein to the reaction mixture.

Reconstitution of adenylate cyclase. Components of adenylate cyclase were reconstituted by adapting the method of Ross and Gilman (25) as described previously (7). Lubrol PX extracts of pigeon erythrocytes were mixed with 20 μg of cyc$^-$ membranes in a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 0.2 mM EGTA, 2 mM 2-mercaptoethanol, bovine serum albumin (0.1 mg/ml), 10 mM creatine phosphate, creatine phosphokinase (10 units/ml), and 0.4 mM ATP. After a 20-min incubation at 30°, [^{32}P]ATP (10^6 cpm) was added to each tube to give a specific activity of 25 cpm/pmole in a final volume of 100 μl. The reaction mixture was incubated for an additional 30 min at 30° and terminated by the addition of 1 ml of 1% SDS. Cyclic AMP was purified by sequential chromatography on Dowex and alumina (26).

Cholera toxin treatment of membranes. Pigeon eryth-

rocyte membranes from control and desensitized cells were washed by centrifugation in 500 mM potassium phosphate (pH 7.0) and resuspended (5 mg of protein per milliliter) in 250 mM potassium phosphate (pH 7.0), 20 mM thymidine, 20 mM arginine, 400 μM ATP, 100 μM GTP, cholera toxin (10 μg/ml) [activated with 20 mM dithiothreitol (23)], and 25 μM [^{32}P]NAD$^+$ (5 Ci/mmole). The labeling proceeded at 30° for 30 min and was terminated by diluting in 10 ml of ice-cold 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM 2-mercaptoethanol (membrane buffer); the mixture was centrifuged and resuspended in membrane buffer at 5 mg of membrane protein per milliliter.

SDS-polyacrylamide gel electrophoresis and autoradiography. Membranes in 1% SDS and 5% 2-mercaptoethanol were boiled for 5 min, and 70% glycerol was added to a final concentration of 8% (w/v). One-dimensional SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (27), as described by O'Farrell (28), using either 10% or 12.5% acrylamide concentrations in the separating gel. Gels were stained and fixed in 2% Coomassie blue, 50% acetic acid, and 10% ethanol, then destained in 10% acetic acid and 10% ethanol. Dried gels were used to expose Kodak X-Omat R film with Dupont lightning-plus intensifying screens at −80°.

Protein determinations. Protein was determined by the method of Lowry *et al.* (29). SDS (0.7% final concentration) was included in assays of protein samples containing Lubrol PX. Bovine serum albumin was used as the standard.

Because it was critical to treat membranes from control and desensitized cells identically, all procedures of each particular experiment were carried out on control and desensitized material simultaneously. After any steps in a procedure which might have resulted in an alteration in protein concentration of the material, protein determinations were made and the values for control and desensitized material were equalized.

Materials. [^{3}H]DHA, [^{32}P]NAD, and [^{32}P]ATP were purchased from New England Nuclear Corporation (Boston, Mass.). Trypsin was purchased from Worthington Biochemicals (Freehold, N.J.). Cholera toxin was obtained from Schwarz/Mann (Orangeburg, N.Y.). Lubrol PX, DL-isoproterenol, DL-propranolol, and aprotinin were purchased from Sigma Chemical Company (St. Louis, Mo.). GTPγS was obtained from Boehringer Mannheim (Indianapolis, Inc.). All other materials were of reagent grade.

RESULTS

The adenylate cyclase activities of membranes prepared from desensitized and control pigeon erythrocytes are shown in Table 1. Prior exposure of cells to isoproterenol resulted in adenylate cyclase activities which were decreased under all assay conditions. Propranolol included in the incubation with isoproterenol prevented the desensitizing effects of isoproterenol (data not shown). The desensitization of agonist-stimulated adenylate cyclase activity varied from approximately 40% to 60% of control values when either GTP or GTPγS was used in the assay. Fluoride-stimulated levels of activity in desensitized membranes showed decreases of 30%–40% from those of control membranes. Desensitized and control adenylate cyclase activities exhibited the same K_{act} for isoproterenol and GTPγS.

TABLE 1

Desensitization of pigeon erythrocyte adenylate cyclase

Pigeon erythrocytes were harvested, washed, and incubated in the presence of 10 μM isoproterenol (desensitized) or with no addition (control). Membranes were then prepared from these cells and adenylate cyclase activities were determined in the absence (basal) or presence of the indicated additions as detailed under Materials and Methods. The values represent the means of triplicate determinations ± standard deviations and are representative of three experiments using three separate membrane preparations (% of control).

Addition	Adenylate cyclase activity	
	Control	Desensitized
	pmoles/min/mg membrane protein	
Basal	1.8	1.1
Isoproterenol	2.0	1.2
GTP	1.6	0.16
GTPγS	43.7 ± 2.9	14.2 ± 0.6[a] (32)
Isoproterenol + GTP	5.2 ± 0.5	2.2 ± 1.8[a] (42)
Isoproterenol + GTPγS	93.2 ± 2.1	46.7 ± 0.7[a] (50)
NaF	57.1 ± 2.4	34.6 ± 1.8[a] (60)

[a] Significantly different from control ($p < 0.01$ by paired Student's *t*-test).

Figure 1 demonstrates that the affinity and number of [^{3}H]DHA binding sites in desensitized pigeon erythrocyte membranes were similar to those found in control membranes. Similar findings, which have been reported in turkey erythrocytes (14), have been interpreted as indicating that the lesion responsible for the desensitized state is distal to the level of hormone-receptor interaction.

Pretreatment of pigeon erythrocyte membranes with isoproterenol and GMP followed by thorough washing presumably results in the release of GDP and the production of an empty guanine nucleotide regulatory site (6, 9). This site is then accessible for GTP binding. In Lubrol PX extracts of membranes solubilized without isoproterenol/GMP pretreatment, GTPγS was unable to activate adenylate cyclase appreciably (Table 2). Adenylate cyclase activity in Lubrol PX extracts of isoproterenol plus GMP-pretreated membranes was markedly activated by GTPγS. Extracts from desensitized membranes exhibited approximately 50% of the GTPγS and fluoride-stimulated adenylate cyclase activities of control extracts (Table 2). Figure 2 demonstrates that the decreased adenylate cyclase activity in desensitized Lubrol-PX extracts was not due to a change in the K_{act} for GTPγS.

Figure 3 represents the results of an experiment designed to demonstrate functional differences between G-proteins solubilized from desensitized and control membranes. When Lubrol extracts from control and desensitized membranes not pretreated with isoproterenol and GMP (so that no adenylate cyclase activity could be measured in the extract) were mixed with cyc$^-$ S49 membranes, no difference was found in the reconstituted adenylate cyclase activity. The concentration of active G-protein used in the reconstitution experiment was varied by diluting the active Lubrol extracts with identical

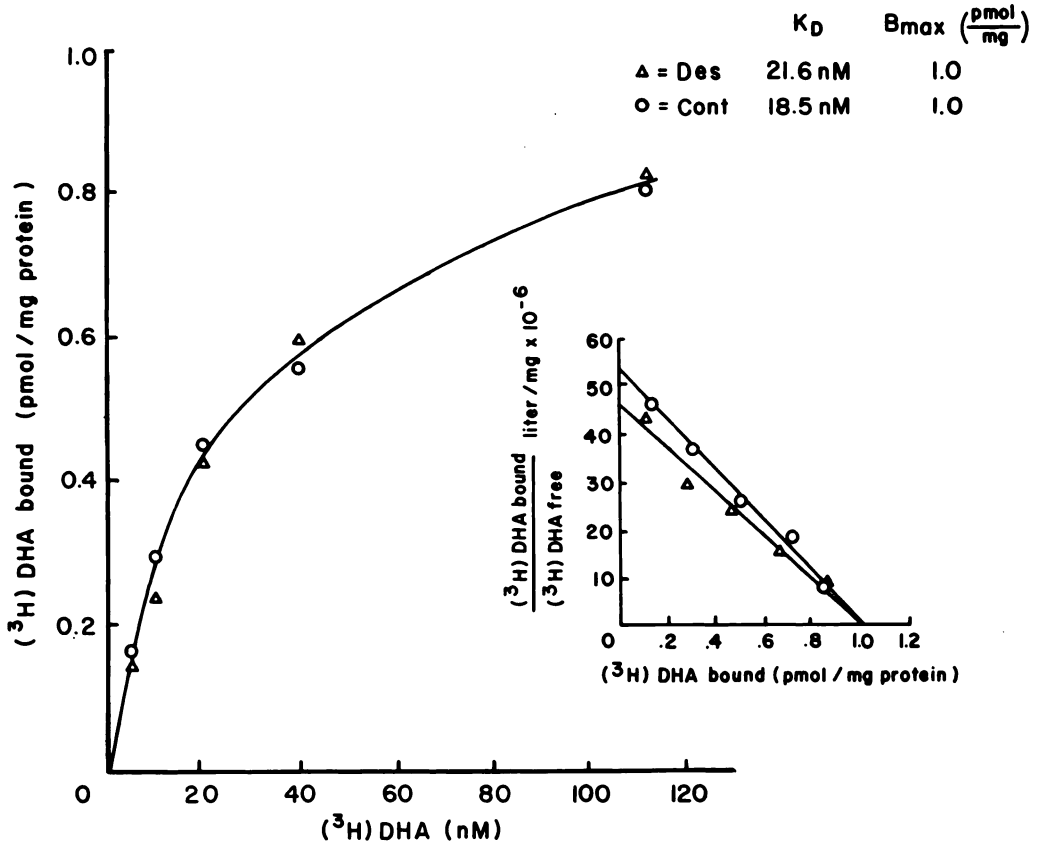


FIG. 1. *Specific binding of [^{3}H]DHA to membranes from control and desensitized pigeon erythrocytes*

Membranes from control and desensitized erythrocytes were washed once in binding buffer by dilution and centrifugation. The pelleted membranes were resuspended in binding buffer and used to start the binding reaction as detailed under Materials and Methods. The specific binding of [^{3}H]DHA to control (○) and desensitized (△) membranes is plotted as a function of [^{3}H]DHA concentration. The *inset* represents the Scatchard analysis of these results. Each *point* represents the mean of duplicate determinations. No significant differences were found in the K_D or B_{max} values for control and desensitized preparations.

heat-inactivated extracts. Fluoride-stimulated cyc$^-$ membranes reconstituted with Lubrol PX extracts from either desensitized or control membranes were identical. Figure 3B demonstrates that reconstituted adenylate cyclase activity was dependent on the active Lubrol extract concentration. It is also evident from Fig. 3B that the cyc$^-$ reconstitution system for assaying G-protein function was sensitive enough to detect easily a 50% change in G-protein activity. Nevertheless, the activity of Lubrol PX extracts of desensitized pigeon erythrocyte membranes was identical with that from control membranes (Fig. 3A and B).

Recently, we described techniques which provide the ability to measure conformational changes of the pigeon erythrocyte G-protein mediated by guanine nucleotides and hormone-receptor interactions (21). Partial tryptic digestion and peptide mapping of cholera toxin-labeled G-protein indicate that guanine triphosphate induces a

TABLE 2

Adenylate cyclase activities of Lubrol extracts of membranes from desensitized and control pigeon erythrocytes

Membranes were prepared from control and desensitized pigeon erythrocytes as in Table 1. The preparations were split and incubated (1 mg of membrane protein per milliliter) in membrane buffer alone or membrane buffer containing 50 μM isoproterenol + 1 mM GMP for 30 min at 37°. The membranes were pelleted and washed by centrifugation once with membrane buffer and three times with solubilization buffer. Adenylate cyclase activities of Lubrol PX extracts of these membranes were determined as detailed under Materials and Methods. The values represent the means of three determinations ± standard deviations and are representative of three experiments using three separate membrane preparations.

Addition to assay	Membrane treatment			
	Isoproterenol/GMP		Membrane buffer	
	Control	Desensitized	Control	Desensitized
	pmol/min/mg protein			
None	1.2	0.9	0.9	1.1
Isoproterenol	1.1	0.9	1.2	0.9
GTPγS	208 ± 5.1	110 ± 5.6[a]	1.0	0.8
Isoproterenol + GTPγS	212 ± 6.1	112 ± 4.8[a]	0.9	0.8
NaF	187 ± 5.0	105 ± 6.1[a]	60.0 ± 2	47.0 ± 2%[a]

[a] Significantly different from control ($p < 0.01$ by paired Student's t test).

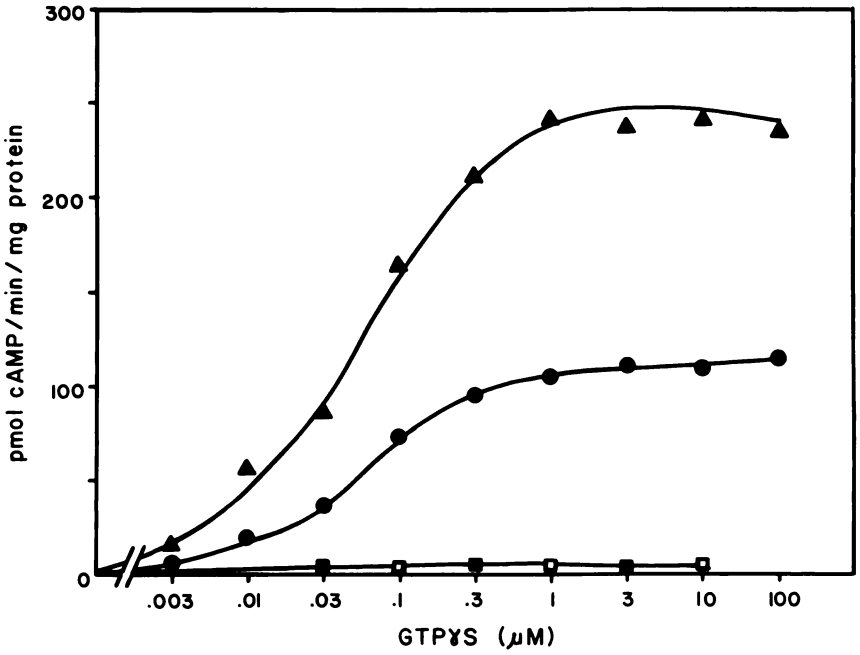


FIG. 2. *Adenylate cyclase activity of Lubrol PX-solubilized, desensitized, and control pigeon erythrocyte membranes*

Desensitized and control pigeon erythrocyte membranes (0.5 mg of protein per milliliter) were incubated for 30 min at 37° in membrane buffer alone or with 50 μM isoproterenol + 1 mM GMP. Membranes were pelleted and washed by centrifugation once with membrane buffer and three times with solubilization buffer. Lubrol PX extracts of the final pellets were assayed for adenylate cyclase activity as detailed under Materials and Methods with the inclusion of the indicated GTPγS concentrations. Extracts from control membranes(▲——▲, isoproterenol/GMP-pretreated; □——□, control preincubation); extracts from desensitized membranes (●——●, isoproterenol/GMP-pretreated; ■——■, control preincubation). The values represent the means of three determinations.

specific conformation on the G-protein and that hormone increases the rate of the guanine triphosphate-mediated conformational change (21). Receptor-hormone interactions in the absence of guanine triphosphate induce changes in tryptic digest patterns distinguishable from peptide maps in the presence of guanine triphosphate (21). Figure 4 is representative of the types of data obtained from these studies. Limited tryptic digestion of membranes in the absence of additions yielded a profile containing a single major band of M_r = 42,000 (*lane 1*). This band corresponded to the single band in nontrypsinized preparations (not shown). Tryptic digests of membranes exposed to isoproterenol resulted in a profile containing the M_r = 42,000 protein and a specific M_r = 38,000 peptide fragment (*lane 2*). The appearance of a specific M_r = 41,000 fragment was the result of tryptic digestion of membranes after incubation with GTPγS (*lanes 3* and *4*). The presence of isoproterenol increased the rate of appearance of the GTPγS-specific M_r = 41,000 tryptic peptide (*lane 4*).

Peptide mapping of isoproterenol-induced conformational states of the G-protein from control and desensitized pigeon erythrocyte membranes in the absence of added guanine nucleotides is shown in Fig. 5. Densitometric scans of the peptide maps clearly showed the M_r = 38,000 peptide generated by limited tryptic digestion. However, the percentage of the total labeled material found in the M_r = 38,000 fragment generated in desensitized membranes was consistently 60% less than that found in control membranes.

The generation of the specific M_r = 41,000 fragment in control and desensitized membrane preparations after incubation with GTPγS and isoproterenol is shown in Fig. 6A. Densitometric scans clearly showed a time-dependent increase in the M_r = 41,000 fragment generated in control and desensitized membranes. The rate and extent of the M_r = 42,000 to M_r = 41,000 conversion differed in desensitized and control membranes. Figure 6B compares the fraction of cholera toxin-labeled material found in the M_r = 41,000 fragment after the indicated times of incubation with isoproterenol and GTPγS. Desensitized membranes exhibited delay in the appearance of the M_r = 41,000 fragment, indicating a slower initial rate of conversion in desensitized membranes from that of control membranes. A plateau in the conversion of the M_r = 42,000 protein to the M_r = 41,000 M_r fragment was reached after 20 min of incubation in both control and desensitized membranes. Using this digestion protocol, the maximal percentage conversion found in control membranes was twice that observed in desensitized membranes (Fig. 6B). Longer incubations with GTPγS and isoproterenol did not alter the percentage tryptic conversion in desensitized membranes relative to controls.

Isoproterenol greatly increased the rate at which GTPγS induced the conversion of M_r = 42,000 to M_r = 41,000 (Fig. 6B). In the absence of isoproterenol, the percent conversion induced by GTPγS in control membranes slowly increased with time. By 30 min, the fraction of total cholera toxin labeled material found in the M_r = 41,000 was 9% of total label. The M_r = 41,000 fragment was not detected in tryptic digests of desensitized membranes incubated with GTPγS (Fig. 6B).

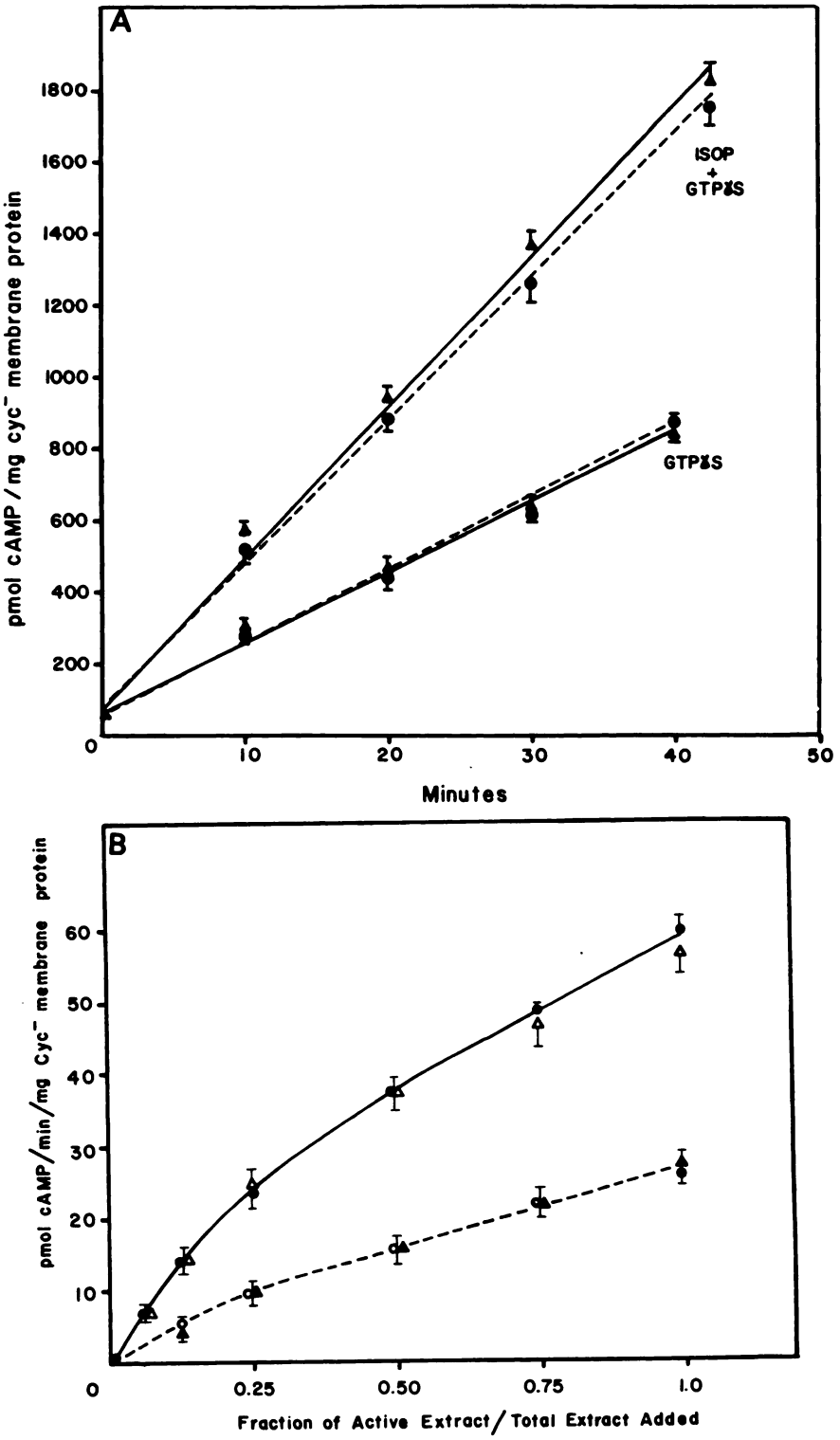


FIG. 3. *Reconstitution of cyc⁻* S49 adenylate cyclase by Lubrol PX extracts of control and desensitized pigeon erythrocyte membranes

Desensitized and control pigeon erythrocyte membranes were solubilized in Lubrol PX and the extracts were used in the reconstitution of cyc⁻ membrane adenylate cyclase as detailed under Materials and Methods.

A. Undiluted Lubrol PX extracts of control membranes (▲——▲) or desensitized membranes (●- - -●) were used for reconstituting cyc⁻ membrane adenylate cyclase activity in the presence of GTPγS (100 μM) with or without isoproterenol (10 μM) as indicated. At the indicated times after the addition of [^{32}P]ATP the reaction was stopped and the generated cyclic AMP determined.

B. Lubrol PX extracts of control membranes (●, ○) or desensitized membranes (▲, △) were diluted with identical Lubrol PX extracts (which had been boiled for 10 min) to give the indicated fractions of active extract/total extract added. These dilutions were then used to reconstitute cyc⁻ membrane adenylate cyclase activity in the presence of GTPγS (100 μM) alone (○, △) or with isoproterenol (10 μM; ●, △) in a 30-min incubation with [^{32}P]ATP. All data *points* represent the means of five determinations. The *bars* represent the standard deviations.

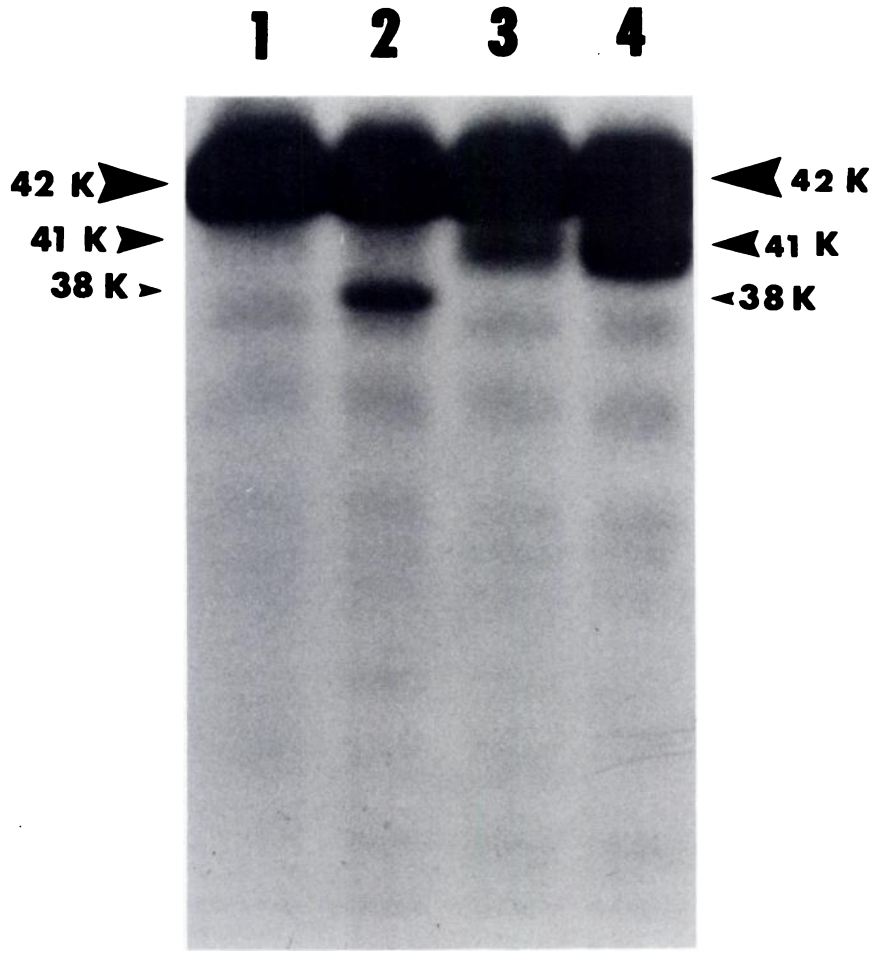


FIG. 4. *Peptide mapping of trypsin fragments of the protein specifically labeled by cholera toxin in pigeon erythrocyte membranes*

Pigeon erythrocyte membranes were labeled with [^{32}]NAD$^+$ and cholera toxin and washed by centrifugation as described under Materials and Methods. The membranes were resuspended (5 mg of membrane protein per milliliter) in membrane buffer and incubated for 20 min at 30° with no addition, *lane 1*; isoproterenol (10 μM), *lane 2*; GTPγS (100 μM), *lane 3*; or isoproterenol and GTPγS, *lane 4*. Trypsin (final concentration of 5 μg/ml) was then added to the membrane resuspensions and digestion proceeded for 12 min at 22°. Proteolysis was stopped by the addition of aprotinin, samples were prepared and electrophoresed in 10% polyacrylamide gels, and autoradiography was performed as detailed under Materials and Methods. The autoradiography shown was the result of a 72-hr exposure to the dried gel. M_r = 42,000, *42K*; M_r = 41,000, *41K* (GTPγS-specific fragment); M_r = 38,000, *38K* (isoproterenol-specific fragment).

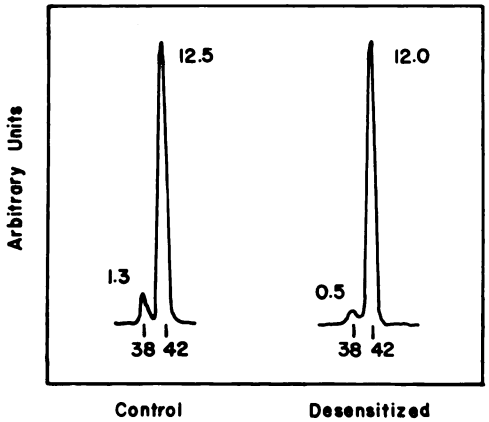
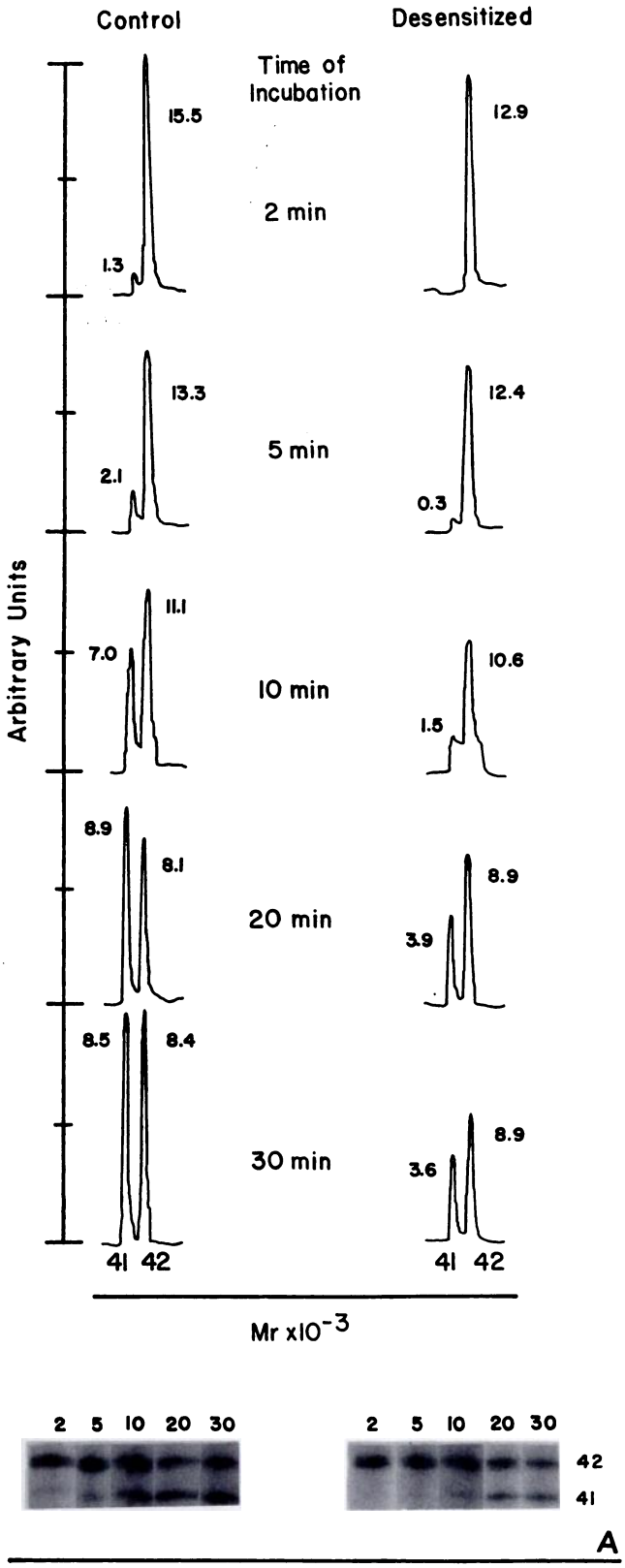


FIG. 5. *Densitometric scans of tryptic digests of the cholera toxin-labeled protein from desensitized and control pigeon erythrocyte membranes incubated with isoproterenol*

Desensitized and control pigeon erythrocyte membranes were labeled with cholera toxin and [^{32}P]NAD$^+$ as detailed under Materials and Methods. The washed membranes were resuspended in membrane buffer (5 mg of membrane protein per milliliter) and incubated in the presence of 10 μM isoproterenol for 25 min at 30°. Trypsin (final concentration 5 μg/ml) was then added to the membrane suspension for 12 min at 22° before proteolysis was stopped by the addition of aprotinin. The samples were then prepared for and electrophoresed on 15% SDS-acrylamide gels as detailed under Materials and Methods. After staining and drying, the gels were autoradiographed for varying lengths of time (12–72 hr). Autoradiographs in which the densities of both the M_r = 42,000 and M_r = 38,000 bands were linear with both exposure time and protein were used for the densitometric scans shown. The relative mobilities of the bands are labeled below their respective density peaks: M_r = 42,000, *42*; M_r = 38,000, *38*. The *numbers* along the *curves* represent the areas beneath the respective peaks (arbitrary units). The areas presented are means of the areas determined from three scans of the indicated preparations. The standard deviations for all values were less than 5% of the mean. These values are representative of two experiments.

DISCUSSION

As measured by partial tryptic digestion of the labeled M_r = 42,000 G-protein subunit, three differences in the conformational states of the G-protein are observed in



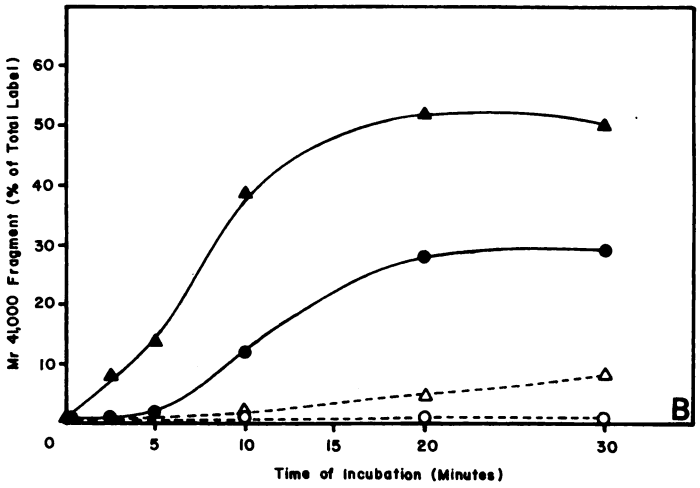


FIG. 6. *Quantitation of peptide maps from tryptic digests of the cholera toxin-labeled protein from desensitized and control pigeon erythrocyte membranes incubated with GTPγS and isoproterenol*

Desensitized and control pigeon membranes were labeled with cholera toxin and [^{32}P]NAD$^+$ as detailed under Materials and Methods. The washed membranes were resuspended in membrane buffer (5 mg of membrane protein per milliliter) and incubated in the presence of 100 μM GTPγS and 10 μM isoproterenol for 0, 2, 5, 10, 20, and 30 min at 30°. At the indicated times, trypsin (final concentration of 25 μg/ml) was added to the membrane suspension, which was then incubated for 12 min at 22° before proteolysis was stopped by aprotinin. The samples were prepared for and electrophoresed on 12.5% SDS polyacrylamide gels as detailed under Materials and Methods. After staining and drying, the gels were autoradiographed for varying lengths of time (8–24 hr).

A. Autoradiographs in which the densities of both the M_r = 42,000 and M_r = 41,000 bands were linear with exposure time and protein were used for the densitometric scans and are shown under their respective series of scans. The relative mobilities of the bands are labeled below their respective density peaks: M_r = 42,000, *42*; M_r = 41,000, *41*. The *numbers* beside the *curves* represent the areas beneath the respective peaks (arbitrary units). The areas presented are the means of the areas determined from three scans of identical lanes. The standard deviations for all values were less than 10% of the mean. These values are representative of two experiments.

B. The density of the M_r = 41,000 band expressed as the percentage of the total cholera toxin specific label is plotted as a function of time of incubation of isoproterenol and GTPγS with control (▲——▲) and desensitized (●——●) pigeon erythrocyte membranes, and GTPγS incubated with control (△- - -△) and desensitized (○- - -○) pigeon erythrocyte membranes.

membranes from desensitized versus control erythrocytes: (a) isoproterenol has a diminished ability to alter the conformation of the G-protein in desensitized membranes (Fig. 5); (b) the onset of the conformational change induced by GTPγS is delayed in desensitized preparations, and the lag occurs in the presence or absence of isoproterenol (Fig. 6); and (c) the fraction of G-proteins found in the GTPγS-specific conformation is significantly decreased in desensitized versus control membranes incubated with GTPγS and isoproterenol (Fig. 6). These findings together provide the first structural evidence that hormone-receptor interactions in desensitized membranes have a decreased ability to alter the conformation of the G-protein. The differences in the conformational states of the G-protein in desensitized and control membranes are related to differences in the heterotropic displacement of GDP by hormone, which in turn allows binding GTP resulting in activation of adenylate cyclase.

Incubation of intact, non-desensitized membranes with GTPγS alone results in a small conversion of total G-protein to a state in which trypsin treatment generates the M_r = 41,000 peptide (Fig. 6B). The amount of M_r = 41,000 peptide generated is small but easily quantitated by autoradiography and densitometry. The fact that there is a significant difference in tryptic generation of the M_r = 41,000 peptide induced by GTPγS in desensitized versus control membranes indicates that GDP exchange at the G-protein regulatory site is altered. However, reconstitution experiments indicate that the G-protein from either membrane source gives identical activities when mixed with cyc^- membranes. These data suggest that a cyclase component other than the G-protein and independent of hormone is influencing GDP exchange at the G-protein regulatory site. These findings are consistent with the kinetic data of Simpson and Pfeuffer (30), who demonstrated an increased lag and a decreased ability of GPP(NH)p to activate adenylate cyclase in desensitized pigeon erythrocyte membranes, suggesting an alteration in GDP exchange at the regulatory site.

The conformational change induced by GTPγS and isoproterenol as quantitated in control and desensitized membranes (Fig. 6A and B) supports the notion of a constraint on the G-protein affecting GDP exchange in desensitized membranes. Desensitized membranes show a lag in onset of tryptic formation of the M_r = 41,000 peptide as well as a decreased maximal percentage conversion of the M_r = 42,000 to the M_r = 41,000 peptide. The total tryptic conversion plateau is reached after a 20-min incubation with isoproterenol and GTPγS. If desensitization involved simply removal of some fraction of receptors from the active pool by altering their coupling, then the ability of isoproterenol to enhance GTPγS-induced tryptic generation of the M_r = 41,000 peptide should be slower in onset but eventually reach the same level of conversion as in control membranes. This is clearly not the case with desensitized membranes, where the percentage conversion to the M_r = 41,000 peptide is about 50% of control values even after long incubations with isoproterenol and GTPγS.

The loss of fluoride activation in the desensitized membranes may be also related to the alteration affecting GDP exchange. A loss of fluoride stimulation has been observed in desensitized turkey erythrocyte membranes (14), but not in membranes from several other cell types (13, 31, 32). A possible explanation for this difference lies in the inherent properties of the avian erythrocyte G-protein and its "exaggerated" tight binding of GDP. It is not difficult to imagine how constraints on the G-protein affecting its ability to release GDP and bind GTPγS could also inhibit fluoride action. This notion is consistent with the findings of Downs *et al.* (33) that the nature of the guanine nucleotide bound to the G-protein in turkey erythrocyte membranes influences fluoride stimulation. In systems where GDP is not so tightly bound, so that the rate of dissociation of GDP is faster, GTP analogues and fluoride could stimulate in desensitized membranes in a manner similar to controls. We cannot test this idea directly by conformational change studies of the G-protein using partial tryptic digestion because cholera toxin pretreatment inhibits fluoride activation.

The nature of the alteration which affects coupling and GDP exchange in pigeon erythrocytes after desensitization is unknown. Whether the altered site is a domain of the hormone receptor or an additional component also present in cyc^- S49 membranes which interacts with the G-protein to regulate coupling to specific receptors is yet to be determined. The ability to measure these effects by partial tryptic digestion of labeled G-proteins combined with reconstitution protocols provides a new approach for studying these regulatory processes.

ACKNOWLEDGMENT

We thank Ms. Nancy Johnson for assistance in preparing the manuscript.

REFERENCES

1. Pfeuffer, T. Guanine nucleotide-controlled interaction between components of adenylate cyclase. *F. E. B. S. Lett.* **101:** 85–89 (1979).
2. Ross, E. M., A. C. Howlett, K. M. Ferguson, and A. G. Gilman. Reconstitution of hormone-sensitive adenylate cyclase activity with resolved components of the enzyme. *J. Biol. Chem.* **253:**6401–6412 (1978).
3. Johnson, G. L., H. R. Kaslow, and H. R. Bourne. Reconstitution of cholera toxin activated adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* **75:**3113–3117 (1978).
4. Cassel, D., and Z. Selinger. Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. *Proc. Natl. Acad. Sci. U. S. A.* **74:**3307–3311 (1977).
5. Howlett, A. C., P. C. Sternweis, B. A. Macik, P. M. Van Arsdale, and A. G. Gilman. Reconstitution of catacholamine-sensitive adenylate cyclase: association of a regulatory component of the enzyme with membranes containing the catalytic protein and β-adrenergic receptors. *J. Biol. Chem.* **254:**2287–2295 (1979).
6. Cassel, D., and Z. Selinger. Mechanism of adenylate cyclase activation through the β-adrenergic receptor: catecholamine induced displacement of bound GDP by GTP. *Proc. Natl. Acad. Sci. U. S. A.* **75:**4155–4159 (1978).
7. Kaslow, H. R., Z. Farfel, G. L. Johnson, and H. R. Bourne. Adenylate cyclase assembled *in vitro*: cholera toxin substrates determine different patterns of regulation by isoproterenol and guanosine 5′-triphosphate. *Mol. Pharmacol.* **15:**472–483 (1979).
8. Sevilla, M., M. L. Steer, and A. Levitzki. Synergistic activation of adenylate cyclase by guanylyl imidodiphosphate and epinephrine. *Biochemistry* **15:**3493–3499 (1976).
9. Lad, P. M., T. B. Nielsen, M. S. Preston, and M. Rodbell. The role of guanine nucleotide exchange reaction in the regulation of the β-adrenergic receptor and in the actions of catecholamines and cholera toxin on adenylate cyclase in turkey erythrocyte membranes. *J. Biol. Chem.* **255:**988–995 (1980).
10. Pfeuffer, T. GTP-binding proteins in membranes and control of adenylate cyclase activity. *J. Biol. Chem.* **252:**7224–7234 (1977).
11. Mickey, J., R. Tate, and R. J. Lefkowitz. Subsensitivity of adenylate cyclase

and decreased β-adrenergic receptor binding after chronic exposure to (−)-isoproterenol in vitro. *J. Biol. Chem.* **250**:5727–5729 (1975).
12. Nordeen, S. K., and D. A. Young. Refractoriness of the cyclic AMP response to adenosine and prostaglandin E_1 in thymic lymphocytes. *J. Biol. Chem.* **253**:1234–1239 (1978).
13. Johnson, G. L., B. B. Wolf, T. K. Harden, P. R. Molinoff, and J. P. Perkins. Role of β-adrenergic receptors in catecholamine-induced desensitization of adenylate cyclase in human astrocytoma cells. *J. Biol. Chem.* **253**:1472–1480 (1978).
14. Hoffman, B. B., D. Mullikin-Kilpatrick, and R. J. Lefkowitz. Desensitization of β-adrenergic stimulated adenylate cyclase in turkey erythrocyte. *J. Cyclic Nucleotide Res.* **5**:355–366 (1979).
15. Bockaert, J., M. Hunzicker-Dunn, and L. Birnbaumer. Hormone-stimulated desensitization of hormone-dependent adenylate cyclase: dual action of luteinizing hormone on pig Graafian follicle membranes. *J. Biol. Chem.* **251**: 2653–2663 (1976).
16. Anderson, W. B., and C. J. Jaworski. Isoproterenol-induced desensitization of adenylate cyclase responsiveness in a cell-free system. *J. Biol. Chem.* **254**: 4596–4601 (1979).
17. Iyengar, R., P. W. Mintz, T. L. Swartz, and L. Birnbaumer. Divalent cation-induced desensitization of glucagon-stimulated adenylate cyclase in rat liver plasma membrane: GTP-dependent stimulation by glucagon. *J. Biol. Chem.* **255**:11875–11882 (1980).
18. Wessels, M. R., D. Mullikin, and R. J. Lefkowitz. Differences between agonist and antagonist binding following β-adrenergic receptor desensitization. *J. Biol. Chem.* **253**:3371–3373 (1978).
19. Su, Y.-F., T. K. Harden, and J. P. Perkins. Catecholamine-specific desensitization of adenylate cyclase: evidence for a multistep process. *J. Biol. Chem.* **255**:7410–7419 (1980).
20. Pike, L. J., and R. J. Lefkowitz. Activation and desensitization of β-adrenergic receptor-coupled GTPase and adenylate cyclase of frog and turkey erythrocyte membranes. *J. Biol. Chem.* **255**:6860–6867 (1980).
21. Hudson, T. H., J. F. Roeber, and G. L. Johnson. Conformational changes of adenylate cyclase regulatory proteins mediated by guanine nucleotides. *J. Biol. Chem.* **256**:1459–1465 (1981).
22. Hudson, T. H., and G. L. Johnson. Peptide mapping of adenylate cyclase regulatory proteins that are cholera toxin substrates. *J. Biol. Chem.* **255**: 7480–7486 (1980).
23. Johnson, G. L., and H. R. Bourne. Influence of cholera toxin on the regulation of adenylate cyclase by GTP. *Biochem. Biophys. Res. Commun.* **78**:792–798 (1977).
24. Ross, E. M., M. E. Maguire, T. W. Sturgill, R. L. Biltonen, and A. G. Gilman. Relationship between the β-adrenergic receptor and adenylate cyclase: studies of ligand binding and enzyme activity in purified membranes of S49 lymphoma cells. *J. Biol. Chem.* **252**:5761–5775 (1977).
25. Ross, E. M., and A. G. Gilman. Reconstitution of catecholamine-sensitive adenylate cyclase activity: interaction of solubilized components with receptor-replete membranes. *Proc. Natl. Acad. Sci. U. S. A.* **74**:3715–3719 (1977).
26. Salomon, Y., C. Londos, and M. Rodbell. A highly selective adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
27. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–686 (1970).
28. O'Farrell, P. H. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007–4021 (1975).
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
30. Simpson, I. A., and T. Pfeuffer. Functional desensitization of beta-adrenergic receptor of avian erythrocytes by catecholamines and adenosine 3′:5′-phosphate. *Eur. J. Biochem.* **111**:111–116 (1980).
31. Shear, M., P. A. Insel, K. L. Melmon, and P. Coffino. Agonist-specific refractoriness induced by isoproterenol: studies with mutant cells. *J. Biol. Chem.* **251**:7572–7576 (1976).
32. Rapaport, B., and R. J. Adams. Induction of refractoriness to thryotropin stimulation in cultured thyroid cells: dependence on new protein synthesis. *J. Biol. Chem.* **251**:6653–6661 (1976).
33. Downs, R. W., Jr., A. M. Speigel, M. Singer, S. Reen, and G. D. Aurbach. Fluoride stimulation is dependent on the guanine nucleotide regulatory protein. *J. Biol. Chem.* **255**:949–954 (1980).

Send reprint requests to: Dr. Thomas H. Hudson, Section of Physiological Chemistry, Division of Biology and Medicine, Brown University, Providence, R. I. 02912.