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Agonist-promoted coupling of the β-adrenergic receptor with the guanine nucleotide regulatory protein of the adenylate cyclase system

(catecholamines/receptor–effector coupling/transmembrane signaling)

LEE E. LIMBIRD*†, D. MICHAEL GILL‡, AND ROBERT J. LEFKOWITZ†§

†Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710; ‡Department of Molecular Biology and Microbiology, Tufts University, Boston, Massachusetts 02114; and §Howard Hughes Medical Institute, Durham, North Carolina 27710

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ABSTRACT **Binding of the β-adrenergic agonist [^{3}H]hydroxybenzylisoproterenol to the β-adrenergic receptor of rat reticulocyte membranes results in the coupling of the receptor to the guanine nucleotide regulatory protein associated with the adenylate cyclase system. This regulatory component, referred to as the G-protein, was identified by its specific [^{32}P]-ADP-ribosylation catalyzed by cholera toxin. Incubation of [^{32}P]ADP-ribosylated rat reticulocyte membranes with the [^{3}H]hydroxybenzylisoproterenol agonist prior to membrane solubilization and gel exclusion chromatography resulted in the coelution of the 42,000 M_r [^{32}P]ADP-ribosylated G-proteins with the agonist-occupied β-adrenergic receptors. The receptor–G-protein complex was not formed when receptors were unoccupied or occupied with antagonists at the time of solubilization. Incubation of rat reticulocyte membranes with [^{3}H]hydroxybenzylisoproterenol in the presence of guanine nucleotides reversed or prevented the formation of this receptor–G-protein complex. These data provide direct evidence for the molecular interactions promoted by agonist occupancy of β-adrenergic receptors. It is probable that the formation of a receptor–G-protein complex is crucial for catecholamine stimulation of the adenylate cyclase enzyme and, hence, transmembrane information transfer.**

Catecholamine-sensitive adenylate cyclase systems are particularly suitable for investigating receptor–enzyme coupling because both β-adrenergic agonist and antagonist agents are available. Thus, one can focus on those molecular changes uniquely promoted by agonist occupancy of the β-adrenergic receptor because it is only agonists that are capable of stimulating adenylate cyclase and effecting transmembrane information transfer.

In earlier studies, we demonstrated that occupancy of frog erythrocyte β-adrenergic receptors with the radiolabeled agonist [^{3}H]hydroxybenzylisoproterenol ([^{3}H]HBI), but not with antagonists, results in an increase in apparent receptor size, as assessed by gel exclusion chromatography (1). This increase in size was not due to a physical coupling of the receptor and catalytic moieties because these elute independently from gel filtration columns (2).

In addition to the receptor and catalytic moieties, adenylate cyclase-coupled systems contain at least one additional protein, referred to throughout the text as the G-protein, which is responsible for the multiple regulatory effects of guanine nucleotides. Binding of GTP to the G-protein promotes its association with the catalytic moiety (3) and the activation of adenylate cyclase activity. Hydrolysis of the bound GTP results in termination of cyclic AMP synthesis (4, 5). The 42,000 M_r G-protein can be ADP-ribosylated by cholera toxin (6–8). This modification restricts GTP hydrolysis and thereby maintains the adenylate cyclase system in an active configuration. Guanine nucleotides not only are required for stimulation of adenylate cyclase by catecholamines (9, 10), but also decrease the affinity of β-adrenergic receptors for agonists, but not antagonists (11, 12), by accelerating the dissociation of receptor–agonist complexes (10, 13). The possibility that agonist occupancy of β-adrenergic receptors results in receptor–G-protein association, thus accounting for the agonist-induced increase in receptor size, was suggested by observations in frog erythrocyte membranes exposed to 20 mM Mn^{2+} (14). Under these conditions, agonist-promoted changes in receptor size are retained in parallel with the ability of guanine nucleotides to decrease receptor affinity for agonists, whereas the ability of both guanine nucleotides and agonists to stimulate the enzyme is lost.

In this paper we show that agonists indeed promote an association of receptors with the G-protein. For these studies, we used rat reticulocyte membranes, which have a well-characterized catecholamine-sensitive adenylate cyclase system (15–17). We were able to unambiguously identify the G-protein by its specific [^{32}P]ADP-ribosylation with cholera toxin and [^{32}P]NAD (18). We found (*i*) that after solubilization, the ^{32}P-labeled G-protein appears larger in the presence of agonists and coelutes with the [^{3}H]HBI agonist–receptor complex and (*ii*) that guanine nucleotides reverse the agonist-dependent increased sizes both of the receptor and of the regulatory protein. These data provide direct evidence for the molecular events associated with agonist occupancy of β-adrenergic receptors and, hence, with receptor–effector coupling.

METHODS AND MATERIALS

The sources of all radiochemicals and other reagents have been reported previously (6, 18).

Membrane Preparations. The procedures for induction of reticulocytosis and preparation of rat reticulocyte membranes have been described (18). Briefly, washed reticulocytes were lysed by suspension in ice-cold water and homogenization in 7.5 mM Tris·HCl/1.25 mM Mg^{2+}/0.15 mM EDTA, pH 7.65. Lysates were centrifuged for 5 min at 19,000 × *g*; membranes were rocked from the bottom of the tube and washed once without homogenization in 75 mM Tris·HCl/12.5 mM $MgCl_2$/1.5 mM EDTA, pH 7.65. The latter buffer was used for all subsequent membrane washing steps after incubations with cholera toxin or [^{3}H]HBI agonist.

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Abbreviations: [^{3}H]HBI, (±)[^{3}H]hydroxybenzylisoproterenol; [^{3}H]DHA, (−)[^{3}H]dihydroalprenolol; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate.

* Present address: Department of Pharmacology, Vanderbilt University, Nashville, TN 37232.

Cholera-Toxin-Catalyzed ADP-Ribosylation of Rat Reticulocyte Membranes. The details of the exposure of concentrated membrane preparations (19–30 mg/5 ml) to cholera toxin and [^{32}P]NAD$^+$ (6, 18) are described in the figure legends. Control preparations were incubated under similar conditions but without toxin. At the end of this incubation, referred to as the intoxication phase, 50-μl aliquots were diluted with 200 μl of buffer and assayed for adenylate cyclase activity to determine the extent of intoxication in each preparation. Previous studies have demonstrated that cholera-toxin-stimulated increases in GTP-sensitive adenylate cyclase activity are paralleled by proportional decreases in fluoride-stimulated adenylate cyclase activity (6, 7). Thus, the extent of intoxication was calculated from the cyclase activity measured with and without 10 mM sodium fluoride, assuming that fluoride has no further effect on the cyclase activity after full intoxication. Letting F and FT be the cyclase activity measured with fluoride before and after treatment with toxin, and B and BT the basal activities (no fluoride) before and after treatment with toxin, then the fraction of cyclase affected by the toxin was $1 - [(FT-BT) \div (F-B)]$.

Assays. [^{3}H]Dihydroalprenolol (DHA) antagonist binding to column eluates was measured as described (1). The components of the adenylate cyclase incubation (18) and methods for cyclic [^{32}P]AMP product purification (19) have been described. Proteins were determined by the method of Lowry *et al.* (20), with bovine serum albumin as the standard.

RESULTS

Agonist-promoted increase in apparent receptor size reversed by guanine nucleotides

As found earlier for frog erythrocyte membranes (1), agonist occupancy of the β-adrenergic receptors of rat reticulocyte membranes results in an increase in the apparent receptor size. Fig. 1*A* demonstrates that β-adrenergic receptors prelabeled with [^{3}H]HBI prior to solubilization eluted from an AcA34 Ultrogel column earlier than unoccupied receptors, which were identified in the column eluates by the binding of the [^{3}H]DHA antagonist. This size change was not observed when guanine nucleotides were present during the preincubation of membranes with agonist. Thus, as shown in Fig. 1*B*, when membranes were exposed simultaneously to agonist and guanyl-5′-yl imidodiphosphate [Gpp(NH)p], a guanine nucleotide analog resistant to hydrolysis, the elution profile of [^{3}H]HBI–receptor complexes was almost superimposable on the elution profile of unoccupied receptors derived from the same membranes. This similarity justifies the assumption that agonists and antagonists bind to the same population of macromolecules and, thus, that the earlier elution of [^{3}H]HBI in Fig. 1*A* represents a real shift in behavior of the receptor rather than the binding of [^{3}H]HBI to a discrete population of proteins.

Coelution of agonist-labeled receptor with [^{32}P]ADP-ribosylated G-protein

Our ability to specifically label the G-protein with [^{32}P]ADP-ribose, by using the reaction catalyzed by cholera toxin, provided the opportunity to test directly whether the agonist-promoted increase in receptor size represented a receptor–G-protein complex. We confirmed that the principal substrate for cholera toxin in rat reticulocyte membranes is a peptide with the same subunit size (42,000 M_r) as the guanine nucleotide regulatory protein identified in other tissues (ref. 18; see Fig. 3*B*). We therefore incubated rat reticulocyte membranes with [^{32}P]NAD and cholera toxin under conditions appropriate for ADP-ribosylating most of the 42,000 M_r protein. GTP, which

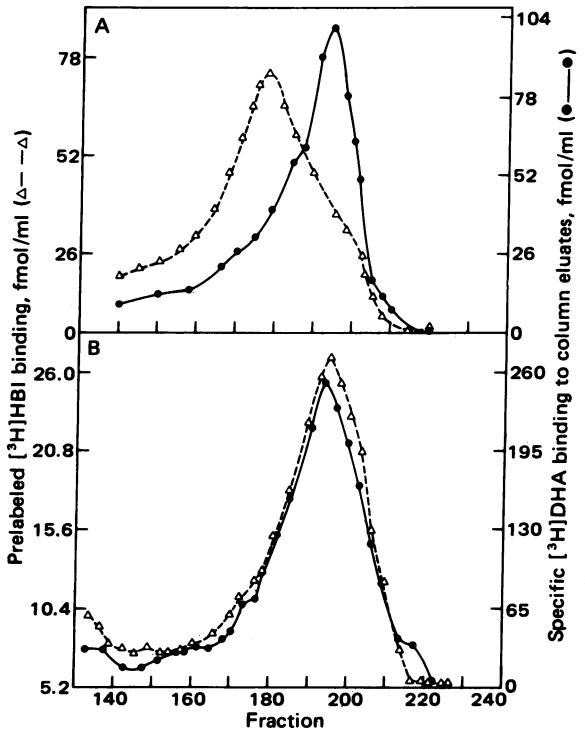

FIG. 1. Prevention or reversal of agonist-promoted increase in apparent β-adrenergic receptor size with guanine nucleotides. (*A*) Elution profile of digitonin-solubilized rat reticulocyte membranes that had been prelabeled with [^{3}H]HBI (16 nM, 15 min at 37°C) in the absence of added guanine nucleotides. Assay of column eluates with [^{3}H]DHA identified unoccupied receptors. (*B*) Elution profile obtained after prelabeling with [^{3}H]HBI in the presence of 0.1 mM Gpp(NH)p. As a consequence of the lower affinity of the β-adrenergic receptor for agonists in the presence of guanine nucleotides, 16 nM [^{3}H]HBI occupies a smaller fraction of the total receptor population when membranes are preincubated in the presence rather than the absence of Gpp(NH)p. Thus, as would be anticipated, the ratio of unoccupied receptors detected by [^{3}H]DHA binding in the eluates to prelabeled [^{3}H]HBI agonist-occupied receptors is greater in *B* than in *A*. Preincubated membranes were washed once, extracted with 0.7% digitonin in 50 mM Tris·HCl/15 mM $MgCl_2$/5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate (EGTA), pH 7.8, and centrifuged at 100,000 × *g* for 60 min. The solubilized material was concentrated about 10-fold by ultrafiltration through an Amicon PM30 membrane and applied to a 2.4 × 90 cm AcA34 Ultrogel column equilibrated with 0.1% digitonin/75 mM Tris·HCl/10 mM Mg^{2+}/2 mM EDTA, pH 7.65. Each fraction was 1.05 ml.

is required during the intoxication phase (21), was then removed by extensive washing. The membranes were next incubated with [^{3}H]HBI, washed again, solubilized with digitonin, and fractionated on an AcA34 Ultrogel column. The data in Fig. 2*A* demonstrate that agonist occupancy of rat reticulocyte β-adrenergic receptors does promote receptor–G-protein association. We have designated the receptor–G-protein complex elution region ($V_e \cong$ fraction 177) as RG_n in the figure. The n subscript in each of the postulated activity regions indicates that the stoichiometries of these multimers have not yet been rigorously determined (see *Discussion*). The elution of the [^{32}P]-ADP-ribosylated G-site in the RG_n region depends markedly on occupation of the receptor by agonist at the time of solubilization; substantially less ^{32}P appears in this region when membranes are not exposed to agonist prior to solubilization (Fig. 2*B*). In the absence of agonist, much more of the G-protein appears small, and elutes in the region designated G_n (Fig. 2*B*). The elution profile of [^{32}P]ADP-ribosylated proteins solubilized from membranes exposed to antagonists is not different from that observed for unoccupied receptors and shown in Fig. 2*B*.

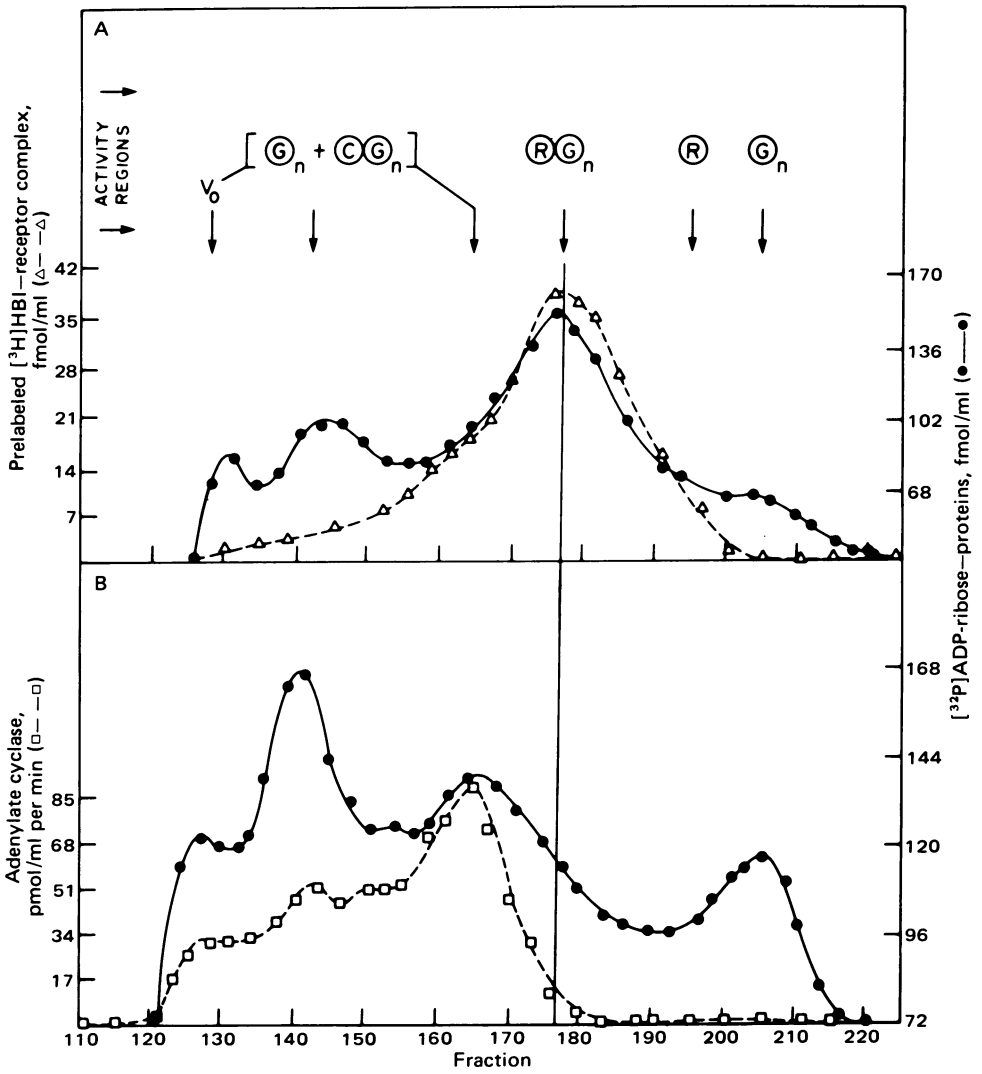

FIG. 2. Gel filtration of digitonin-solubilized [^{32}P]ADP-ribosylated membrane proteins, β-adrenergic receptors, and adenylate cyclase. Each fraction was 1.07 ml. (*A*) Rat reticulocyte membranes (19 mg/5 ml) were exposed for 10 min at 25°C to 50 μg of cholera toxin per ml (preactivated for 10 min at 37°C with 20 mM dithiothreitol), 5 μM [^{32}P]NAD$^+$ (11.3 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels), 50 μM GTP, and a GTP-regenerating system. The cyclase was 69% activated by cholera toxin. The membranes were washed five times with ice-cold buffer, resuspended to 0.8 mg/ml in the presence of 0.3 mM catechol and 0.8 mM ascorbate, and incubated with 16 nM [^{3}H]HBI (15.7 Ci/mmol) for 30 min at 37°C. Membranes were then washed twice more, solubilized with 1% digitonin in 50 mM Tris·HCl/15 mM Mg^{2+}/2 mM EDTA, pH 7.8, concentrated, and applied to a 2.4 × 99 cm AcA34 Ultrogel column equilibrated and eluted with 0.1% digitonin/75 mM Tris·HCl/2 mM EDTA, pH 7.65. (*B*) Rat reticulocyte membranes were incubated with cholera toxin and [^{32}P]NAD$^+$ (15.5 Ci/mmol) as above. The cyclase was 84% activated. The membranes were washed five times before solubilization. In order to allow a direct comparison of the elution profile of adenylate cyclase activity with that of [^{32}P]ADP-ribosylated protein, the solubilized toxin-treated preparation was mixed with a solubilized preparation derived from control membranes preincubated for 90 min at 25°C with 0.1 mM Gpp(NH)p. Adenylate cyclase activity in the eluates was assayed for 30 min at 25°C in the presence of 0.3 mM ATP and 6 mM Mg^{2+}. The elution positions of soluble marker proteins from this column were: ferritin, R_S = 59.3 Å, M_r 456,000, V_e = fraction 167; catalase, R_S = 52.2 Å, M_r 250,000, V_e = fraction 205; IgG, R_S = 51 Å, M_r 166,000, V_e = fraction 210; bovine serum albumin, R_S = 36.3 Å, M_r 67,000, V_e = fraction 254. V_0 = void volume; G = G-protein, C = catalytic moiety of adenylate cyclase; R = β-adrenergic receptor; the subscripts n denote uncertainty about the stoichiometry of G in the various complexes.

[^{32}P]ADP-ribosylated G-proteins elute in regions other than RG_n and G_n with or without agonist occupancy of R. The identity of the components in these other elution positions is discussed later.

Fig. 3*A* demonstrates an intermediate situation. Here, the GTP required for cholera toxin-catalyzed ADP-ribosylation of rat reticulocyte membranes was not entirely removed prior to membrane incubation with the [^{3}H]HBI agonist. As already shown in Fig. 1, occupancy of G-proteins with GTP prevents or reverses formation of the RG complex and the [^{3}H]HBI–receptor complex elutes in the region of smaller molecular size, designated R. Thus, it is important to note in Fig. 3*A* that under those circumstances where [^{3}H]HBI receptor complexes elute in both the RG_n and R activity regions, the [^{32}P]ADP-ribosylated G-proteins coelute only with the RG_n form of the receptor.

Fig. 3*B* shows the autoradiograms of polyacrylamide gel analyses of column eluates pooled from various activity regions. As shown on the right, digitonin solubilized almost exclusively the 42,000 M_r protein, the ADP-ribosylation of which is specifically catalyzed by cholera toxin. The figure verifies that each of the ^{32}P radioactivity peaks indeed represents this 42,000 M_r protein.

As indicated above in Fig. 2*B*, [^{32}P]ADP-ribosylated G-proteins are present in several elution regions whether or not receptors are occupied by the [^{3}H]HBI agonist (Fig. 2*B*). We

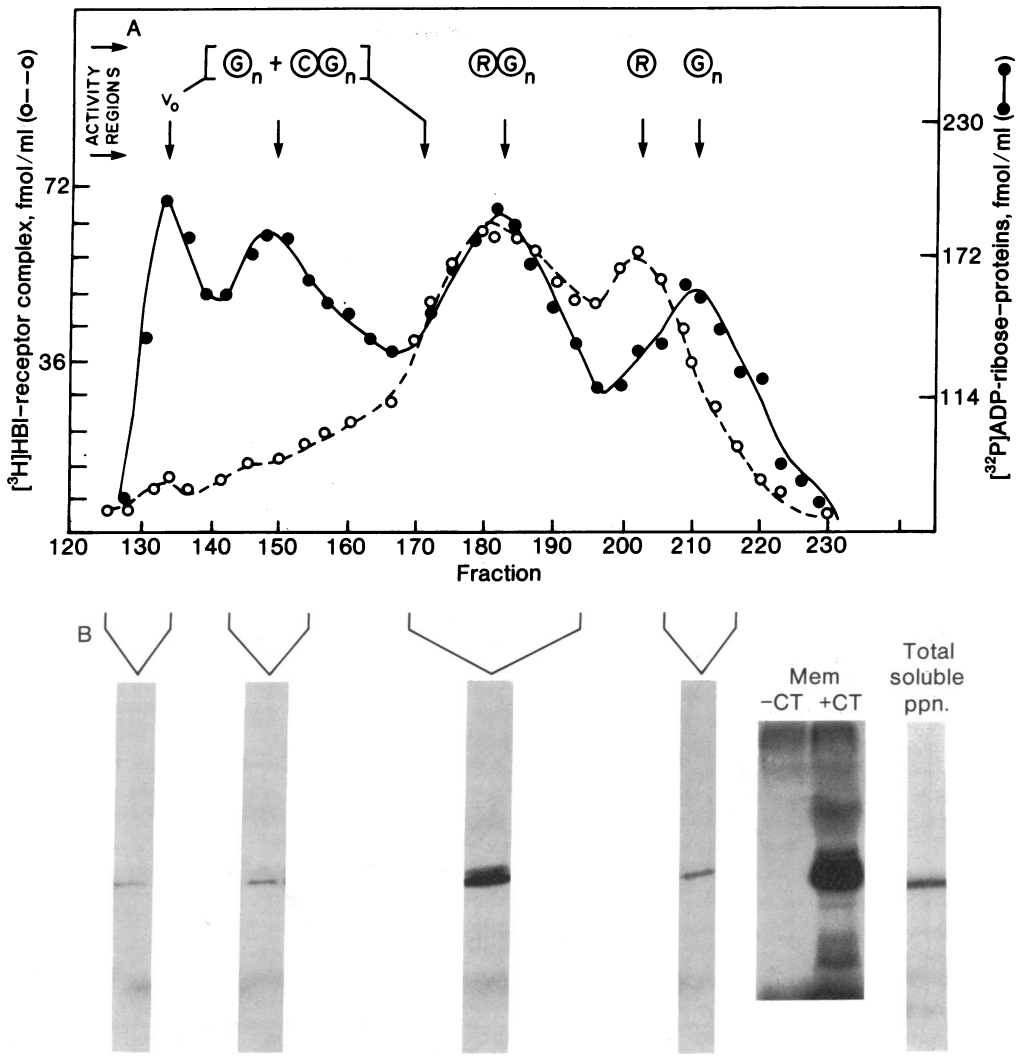

FIG. 3. NaDodSO$_4$/polyacrylamide electrophoresis of AcA34 Ultrogel eluates pooled from peak elution regions of solubilized [^{32}P]ADP-ribosylated rat reticulocyte membrane proteins. (*A*) Rat reticulocyte membranes were preincubated with 5 μM [^{32}P]NAD$^+$, 0.1 mM GTP, and a regenerating system for 10 min at 25°C, resulting in 44% intoxication. Membranes were washed only three times and then incubated with [^{3}H]HBI and fractionated as in the legend for Fig. 2. Incomplete removal of GTP during the washing resulted in the elution of the [^{3}H]HBI–receptor complex in two positions corresponding to RG and R. (*B*) Analysis of the peaks by NaDodSO$_4$/polyacrylamide gel electrophoresis. Fractions from the column were pooled, concentrated by ultrafiltration through an Amicon PM30 membrane at 10 lb./inch^2, boiled for 2 min with 1% NaDodSO$_4$/26 mM 2-mercaptoethanol/100 mM Tris·HCl, pH 6.8, and subjected to electrophoresis in a gradient of 7.5–15% polyacrylamide, with a 5% stacking gel, in the presence of 1% NaDodSO$_4$. [^{32}P]ADP-ribosylated proteins were detected by autoradiography for 7 days with Kodak SB-5 film. On the right are shown the proteins in membranes (Mem) incubated without (−CT) or with (+CT) cholera toxin and in the solubilized preparation applied to the column.

have indicated the possible molecular complexes responsible for these peaks of radioactivity above the curves in Fig. 2*A*. The smallest form of the G-protein ($V_e \cong$ fraction 206) is free of receptor and catalytic activity and is probably an aggregate of G-proteins (G_n) or is complexed with an unidentified component because it appears substantially larger than the peptide size of 42,000. Adenylate cyclase activity elutes in the void volume and in two regions of larger molecular size. The ratio of enzyme activity to ^{32}P-labeled G-proteins varies among these regions and they may therefore represent either various complexes of the catalytic moiety, C, with G-proteins or the coincidental coelution of these CG_n complexes with larger aggregates of G_n. The peak of [^{32}P]ADP-ribosylated proteins eluting with the major peak of catalytic activity ($V_e \cong$ fraction 166) is broader than the peak of catalytic activity (Fig. 2*B*). It is possible that this may be due to a small population of RG_n complexes existing in the absence of added agonist and eluting at approximately fraction 177.

In experiments in which cholera toxin failed to activate adenylate cyclase when assayed after the intoxication phase (e.g., when GTP and a GTP-regenerating system were omitted from the intoxication medium) no 42,000 M_r [^{32}P]ADP-ribosylated proteins were formed and no ^{32}P-labeled proteins were detected in the included volume of the AcA34 columns.

DISCUSSION

The data presented provide direct evidence that agonist occupancy of the β-adrenergic receptor causes a physical coupling of the receptor to the G-protein associated with adenylate cyclase systems. Two pieces of evidence indicate that the G-protein that associates with the receptor modulates receptor affinity for agonist agents. First, guanine nucleotides are able to both reduce receptor affinity for agonists and prevent or reverse formation of the agonist-promoted receptor–G-protein complex. Second, earlier studies in frog erythrocyte membranes exposed to high concentrations of Mn^{2+} demonstrated that the

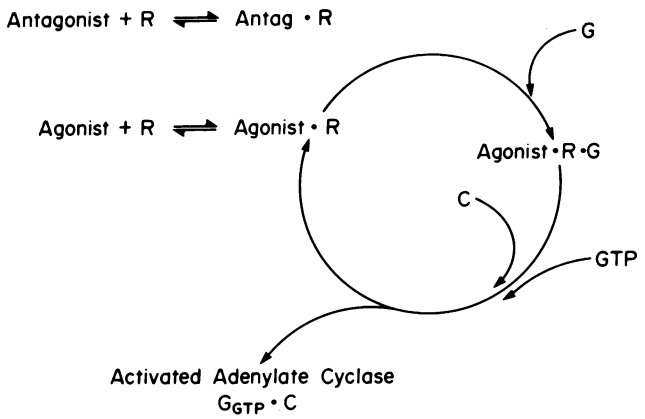

FIG. 4. Representation of the molecular events that translate agonist occupancy of the β-adrenergic receptor to stimulation of adenylate cyclase. Both agonists and antagonists bind to β-adrenergic receptors (R) with high affinity and specificity, but only agonists are capable of promoting the association of the GTP-binding protein (G) with the receptor. The presence of GTP results both in the activation of the catalytic moiety (C) and dissociation of the RG complex. See text for further discussion.

retention of the ability of guanine nucleotides to modulate β-adrenergic receptor affinity for agonists correlates with the retention of the ability of agonists to increase the apparent β-adrenergic receptor size despite the loss of guanine nucleotide and catecholamine stimulation of adenylate cyclase activity under these conditions (14). Likewise, we believe the 42,000 M_r G-protein coeluting with the receptor modifies adenylate cyclase activity because the extent of its ADP-ribosylation by cholera toxin parallels the extent of enhancement of GTP-sensitive enzyme activity (18). Thus, the data suggest that the same G-proteins, or population of proteins, both modify receptor affinity for agonists and allow formation of cyclic AMP by the catalytic moiety. Determination of the precise stoichiometry of the RG_n complex by techniques similar to those used in these studies will require additional information concerning the molar ratio of [^{32}P]ADP-ribose incorporated per mole of 42,000 M_r G-protein as well as hydrodynamic measurements to determine the contribution of detergent and lipid to the size of the molecular complexes detected by gel filtration.

The formation of a receptor–G-protein complex in the presence of agonist seems to be a necessary event in the stimulation of adenylate cyclase, especially since it is not promoted by antagonists. Our data and those of others suggest the sequence of events involved in receptor–effector coupling outlined in Fig. 4. Both agonists and antagonists bind to the receptors, but only agonists promote an association of receptors with the G-protein. The larger complex is stable in the absence of GTP and has a higher affinity for agonist than has the free receptor. GTP is required for the stimulation of adenylate cyclase by catecholamines (9, 10). When GTP occupies the G-protein, two consequences ensue. The catalytically active form of adenylate cyclase (CG_{GTP}) is created (3), and the high-affinity agonist–receptor complex is reversed. It is not clear at present whether these occur simultaneously or sequentially. Formation of cyclic AMP continues until the bound GTP is hydrolyzed to GDP (4, 5). It has been suggested that one role of catecholamines is to promote release of GDP from the G-protein so that the vacated guanine nucleotide-binding site can be occupied by ambient GTP (22). Thus, the formation of an RG complex promoted by agonist occupancy of the receptor provides the molecular association essential for unloading the GDP and permitting reformation of the active CG_{GTP} complex. By these postulates, the G-protein is conceptualized as a communicator between the β-adrenergic receptor and adenylate cyclase enzyme. Current attempts by numerous investigators to isolate, purify, and reconstitute this multicomponent system will, we hope, permit the direct evaluation of these postulates.

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