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INTERACTION OF β-ADRENERGIC RECEPTORS WITH THE INHIBITORY GUANINE NUCLEOTIDE-BINDING PROTEIN OF ADENYLATE CYCLASE IN MEMBRANES PREPARED FROM cyc⁻ S49 LYMPHOMA CELLS*

STEWART N. ABRAMSON,† MICHAEL W. MARTIN,‡ ARLENE R. HUGHES,‡
T. KENDALL HARDEN,‡ KIM A. NEVE,§ DEBRA A. BARRETT‖ and PERRY B. MOLINOFF¶
Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6084, U.S.A.

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Abstract—β-Adrenergic receptors on membranes prepared from L6 myoblasts, wild-type S49 lymphoma cells, and an adenylate cyclase-deficient variant (cyc⁻) of S49 lymphoma cells bind the agonist [^{3}H]hydroxybenzylisoproterenol ([^{3}H]HBI) with high affinity. In each case the agonist [^{3}H]HBI is associated with a larger complex than is the antagonist [^{125}I]iodopindolol, and the binding of [^{3}H]HBI can be inhibited by GTP. These observations suggest that there is an agonist-dependent association of the receptor with a guanine nucleotide-binding protein. The goal of the present experiments was to investigate the possibility that an interaction of β-adrenergic receptors with the inhibitory guanine nucleotide-binding protein of adenylate cyclase was responsible for these observations. Treatment of S49 cells with pertussis toxin decreased the extent of pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of a 41,000-dalton protein, measured *in vitro*, and decreased the inhibition of adenylate cyclase activity observed in the presence of somatostatin or analogues of GTP. Isoproterenol-stimulated adenylate cyclase activity was potentiated following treatment of wild-type S49 cells and L6 myoblasts with pertussis toxin. Although the ability of receptors on membranes prepared from L6 myoblasts to bind the agonist [^{3}H]HBI was not affected by treatment of cells with pertussis toxin, treatment of cyc⁻ S49 cells with pertussis toxin markedly decreased the ability of receptors to bind [^{3}H]HBI. The observed inhibition of the binding of the agonist [^{3}H]HBI to β-adrenergic receptors on membranes prepared from cyc⁻ S49 cells after treatment with pertussis toxin could be explained by an interaction between β-adrenergic receptors and the inhibitory guanine nucleotide-binding protein. Such an interaction may represent a mechanism through which stimulation of the activity of adenylate cyclase by β-adrenergic receptors can be regulated or through which β-adrenergic receptors can affect the activity of cyclic AMP-independent cellular processes.

Hormones and neurotransmitters that stimulate or inhibit the activity of adenylate cyclase do so through activation of the stimulatory (G_s)** or inhibitory (G_i) guanine nucleotide-binding proteins of adenylate cyclase respectively. Activation of G_s and G_i involves dissociation of their alpha and beta-gamma subunits. The activity of adenylate cyclase is increased by a direct action of the alpha subunit of G_s on the catalytic unit of adenylate cyclase. However, G_i is thought to inhibit the activity of adenylate cyclase either by a direct action of the alpha subunit of G_i on the catalytic unit of adenylate cyclase, or as a consequence of an interaction between the beta subunit of G_i and the activated alpha subunit of G_s. The observation that the beta subunits of G_s and G_i are capable of deactivating the alpha subunit of G_s supports the latter hypothesis [1–4]. However, since the adenylate cyclase-deficient variant (cyc⁻) of S49 lymphoma cells does not express G_s [5–8], the observation that adenylate cyclase activity in cyc⁻ S49 cells can be inhibited by endogenous and exogenous G_i supports the hypothesis that the alpha subunit of G_i acts directly on the catalytic unit of adenylate cyclase [9–11]. In addition to mediating inhibition of adenylate cyclase activity by hormones and guanine nucleotides, G_i appears to be involved in attenuation of

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† Recipient of a Predoctoral Fellowship from the Pharmaceutical Manufacturers Association Foundation. Present address: Department of Pharmacology, School of Medicine, University of California-San Diego, La Jolla, CA 92093.

‡ Department of Pharmacology, University of North Carolina, School of Medicine, Chapel Hill, NC 27514.

§ Present address: Psychiatry Service, VA Medical Center, Portland, OR 97207.

‖ Present address: Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0001.

¶ Correspondence should be sent to: Dr. P. B. Molinoff, Professor and Chairman, Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6084.

** Abbreviations: G_s and G_i, the stimulatory and inhibitory guanine nucleotide-binding proteins of adenylate cyclase respectively; [^{3}H]HBI, (±)-[^{3}H]hydroxybenzylisoproterenol; [^{125}I]IPIN, (−)-[^{125}I]iodopindolol; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); Gpp(NH)p, guanosine-5′-(β-imido)triphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; cyc⁻, the adenylate cyclase-deficient variant of the S49 lymphoma cell; DMEM, Dulbecco's modification of Eagle's medium; SDS, sodium dodecyl sulfate; and EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate.

hormone-stimulated adenylate cyclase activity, since stimulation of adenylate cyclase activity by agonists at β-adrenergic receptors can be potentiated by treatment of intact cells with pertussis toxin [12].

Guanine nucleotide-sensitive high-affinity binding of the agonist [^{3}H]HBI to β-adrenergic receptors has been observed in experiments carried out with a variety of cells, and agonist–receptor complexes have been shown to be larger than antagonist–receptor complexes [13–17]. These results were initially thought to reflect the formation of a ternary complex composed of agonist, receptor, and G_s. However, similar results have been obtained in studies carried out with membranes prepared from cyc$^-$ S49 lymphoma cells [15, 16]. Because cyc$^-$ S49 lymphoma cells do not express the alpha subunit of G_s [5–8] but do express functional G_i [9–11], we suggested that the high-affinity binding of [^{3}H]HBI to membranes prepared from cyc$^-$ S49 cells could reflect an association between β-adrenergic receptors and G_i [15, 16].

Ribosylation of G_i by pertussis toxin blocks the ability of receptors to bind agonists with high affinity and also blocks the ability of receptors to inhibit the activity of adenylate cyclase [11, 18–20]. To test the hypothesis that β-adrenergic receptors can interact with G_i in intact membranes, the effects of pertussis toxin on the properties of β-adrenergic receptors from S49 lymphoma cells and L6 myoblasts have been investigated. Isoproterenol-stimulated adenylate cyclase activity of membranes prepared from wild-type S49 cells and L6 myoblasts was potentiated by treatment of intact cells with pertussis toxin, and the binding of [^{3}H]HBI to β-adrenergic receptors on membranes prepared from cyc$^-$ S49 cells decreased after treatment of intact cells with pertussis toxin. These results are consistent with the hypothesis that β-adrenergic receptors can interact with a pertussis toxin-sensitive component, presumably G_i, in membranes prepared from these cells.

MATERIALS AND METHODS

Materials. Racemic [^{3}H]HBI (7.7 to 17.5 Ci/mmol) and [α-^{32}P]ATP (approximately 1000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Carrier-free Na^{125}I (2200 Ci/mmol) was purchased from either New England Nuclear or Amersham (Arlington Heights, IL). Gpp(NH)p and GTPγS were purchased from Boehringer Mannheim (Indianapolis, IN). Forskolin was purchased from Calbiochem (San Diego, CA). (−)-Isoproterenol, NAD, ATP, cyclic AMP, propranolol, and GTP were obtained from the Sigma Chemical Co. (St. Louis, MO). (−)-Pindolol was a gift of Dr. Günter Engel (Pharmaceutical Division, Sandoz Ltd., Basel, Switzerland).

Tissue culture. L6 myoblasts were grown in monolayer culture in 150 mm Petri dishes containing 20 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in a humidified atmosphere of 10% CO_2/90% air at 37° as described by Abramson and Molinoff [15]. Cells were subcultured every 3 or 4 days. To subculture, the cells were detached from the Petri dishes using pancreatic trypsin (0.5 mg/ml) and EDTA (0.2 mg/ml) in phosphate-buffered saline (138 mM NaCl, 4.1 mM KCl, 5.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 11.1 mM glucose, pH 7.4), and dispersed by gentle trituration of the cells against the bottom of the dish. Cells were then transferred into new Petri dishes at a density of approximately 10,000 cells/cm^2. Medium was changed every 2 days, and cells were harvested after 7 days.

S49 lymphoma cells were obtained from the Cell Culture Facility of the University of California, San Francisco, CA, and the phenotypes were periodically substantiated by assays of adenylate cyclase activity using isoproterenol, forskolin, NaF, and $MnCl_2$ as activators of enzyme activity [15]. S49 lymphoma cells were maintained in stationary suspension in a humidified atmosphere of 10% CO_2/90% air at 37°. The density of cells in stock cultures was kept between 1×10^4 and 2×10^6 cells/ml by addition of DMEM supplemented with 10% horse serum that had been filtered through a 0.2-μm sterile filter. The large number of cells needed for experiments with [^{3}H]HBI were obtained by growing cells in spinner flasks and harvesting them when they reached a density of 1 to 3×10^6 cells/ml.

L6 myoblasts and S49 lymphoma cells were treated with pertussis toxin by the addition of a concentrated suspension of toxin 16 hr prior to harvesting the cells and preparing membranes. Pertussis toxin was prepared from *Bordatella pertussis* as previously described [21].

Preparation of cell membranes. Attached L6 myoblasts were rinsed once with 20 mM Na^+-HEPES buffer (pH 7.4) containing 1.0 mM $MgCl_2$ (HEPES/Mg^{2+} buffer), removed from the Petri dishes with a rubber policeman, and homogenized in HEPES/Mg^{2+} buffer with a Brinkmann Polytron. Homogenates were centrifuged at 20,000 *g* for 20 min and resuspended in HEPES/Mg^{2+} buffer for assay with [^{125}I]IPIN or [^{3}H]HBI. Purified membranes were prepared from S49 lymphoma cells as previously described [6, 15]. In some experiments, membranes were used just prior to purification through sucrose density centrifugation.

Radioligand binding assays of β-adrenergic receptors. [^{125}I]IPIN was synthesized from (−)-pindolol and Na^{125}I in the presence of chloramine-T as described by Wolfe and Harden [22]. Membranes prepared from L6 myoblasts or S49 lymphoma cells (4–10 μg protein/assay) were incubated for 120 min at 22° with [^{125}I]IPIN in a total volume of 250 μl of 20 mM Na^+-HEPES (pH 7.4) containing 1.0 mM $MgCl_2$, 500 μM pyrocatechol, and 500 μM ascorbate. Reactions were terminated by the rapid addition of 10 ml of 10 mM Tris–HCl (pH 7.5) containing 1.0 mM $MgCl_2$ at room temperature, followed by filtration through No. 30 Schleicher and Schuell glass-fiber filters. Filters were washed with an additional 10 ml of buffer. Specific binding was defined as the amount of [^{125}I]IPIN bound to the tissue in the absence of a competing ligand minus the amount bound to the tissue in the presence of 50 μM (−)-isoproterenol and generally represented more than 90% of the total binding of [^{125}I]IPIN. The total amount of ligand bound to the tissue was usually less than 10% of the total ligand added to the assay.

Membranes prepared from L6 myoblasts or S49 lymphoma cells (200–400 μg protein/assay) were incubated for 120 min at 22° with [^{3}H]HBI in a total volume of 1.0 ml of 20 mM Na^+-HEPES (pH 7.4) containing 1.0 mM $MgCl_2$, 500 μM pyrocatechol, and 500 μM ascorbate. Reactions were terminated by the rapid addition of 10 ml of 10 mM Tris–HCl (pH 7.5) containing 1.0 mM $MgCl_2$ at room temperature, followed by filtration through Gelman AE glass-fiber filters. Filters were washed with an additional 10 ml of buffer. Specific binding was defined as the amount of [^{3}H]HBI bound to the tissue in the absence of a competing ligand minus the amount bound to the tissue in the presence of 1.0 μM (−)-propranolol. Specific binding of [^{3}H]HBI to β-adrenergic receptors on membranes prepared from L6 myoblasts and cyc$^-$ S49 lymphoma cells represented approximately 80 and 40–60% of total binding, respectively, at the K_d value for the radioligand. The total amount of ligand bound to the tissue was between 10 and 30% of the total amount of [^{3}H]HBI added to the assay.

Measurement of cyclic AMP (cAMP) accumulation in intact cells. S49 lymphoma cells (1 to 2 × 10^6 cells/ml) were incubated with [^{3}H]adenine (1–2 μCi/ml) for 75 min at 37°. Cells were collected by centrifugation at 2500 *g* for 10 min and resuspended in growth medium. Assays with agents that affect the accumulation of cyclic AMP were carried out in a final volume of 3.6 ml of growth medium for 10 min at 37°. Cells were then collected by centrifugation at 2500 *g* for 10 min, and intracellular cyclic AMP was extracted by the addition of 1 ml of 5% (w/v) trichloroacetic acid. [^{3}H]cAMP was separated from [^{3}H]ATP as described by Meeker and Harden [23], using sequential chromatography over Dowex and alumina columns. Cyclic AMP synthesized during the assay was expressed as the percentage of [^{3}H]ATP converted to [^{3}H]cAMP.

Assay of adenylate cyclase activity. The conversion of [α-^{32}P]ATP to [^{32}P]cAMP was determined essentially as described by Salomon *et al.* [24]. Each assay included 10–40 μg of membrane protein in 100 μl of buffer containing 50 mM Na^+-HEPES (pH 7.4), 0.5 mM EGTA, 5 mM cyclic AMP, 0.25 mM ATP, 1.0 mM 3-isobutyl-1-methylxanthine, 30 μM GTP, 1.0 mM $MgCl_2$, 0.1 mg/ml creatine phosphokinase, 3.45 mg/ml phosphocreatine, 2 × 10^6 cpm of [α-^{32}P]ATP, and various drugs. Assays were carried out at 22° for 20 min and were initiated by addition of [α-^{32}P]ATP. Reactions were terminated by the addition of 100 μl of 50 mM Tris–HCl (pH 7.5) containing 10% sodium dodecyl sulfate (SDS) (w/v) and 5.0 mM ATP, and assay tubes were then placed in a boiling water bath for 10 min. [^{3}H]cAMP (approximately 30,000 cpm) was added to the assays as an internal standard for the recovery of [^{32}P]cAMP. The assay volume was brought up to 1 ml, and cyclic AMP was isolated by sequential chromatography on columns containing Dowex AG50W-X4 resin and neutral alumina.

[^{32}P]*ADP-ribosylation of* G_i. [^{32}P]NAD was synthesized by the method of Cassel and Pfeuffer [25]. Purity was assessed by thin-layer chromatography on PEI cellulose. [^{32}P]ADP-ribosylation of proteins was performed by incubating membranes (100–200 μg protein) with or without pertussis toxin (approximately 6 μg) in buffer containing 100 mM Tris–HCl (pH 8.0), 10 μM NAD, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM ATP, 1.0 mM GTP, 0.1 mg/ml creatine phosphokinase, 3.45 mg/ml phosphocreatine, and [^{32}P]NAD (2 to 10 × 10^6 cpm) for 1 hr at 30°. Assays were terminated by the addition of 1 ml of buffer containing 100 mM Tris–HCl (pH 8.0) and 2.5 mM $MgCl_2$ at 4°. Membranes were sedimented by centrifugation in a Beckman Microfuge for 2 min, gently washed with 1 ml of buffer, and recentrifuged. The proteins were solubilized overnight at room temperature in sample buffer containing 1% SDS, 50 mM Tris–HCl (pH 6.8), 1% β-mercaptoethanol, and 10% glycerol and were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 11% or 12.5% acrylamide gels. The gels were stained with Coomassie brilliant blue R-250 for visualization of proteins, and [^{32}P]ADP-ribosylated proteins were visualized by exposure of dried gels to Kodak XAR-5 X-ray film for 2–12 days. Autoradiograms were scanned with a microcomputer-assisted densitometer, and the relative extent of [^{32}P]ADP-ribosylation was quantitated by cutting out and weighing the appropriate peak areas.

Protein determination. Protein was determined by the method of Bradford [26] using bovine serum albumin as a standard.

RESULTS

Incubation of membranes prepared from cyc$^-$ S49 lymphoma cells with [^{32}P]NAD resulted in the incorporation of radioactivity into several proteins (Fig. 1, lane 1). However, only a protein with an apparent molecular weight of 41,000 daltons was selectively [^{32}P]ADP-ribosylated in the presence of pertussis toxin (Fig. 1, lane 2). Treatment of intact cyc$^-$ S49 cells with pertussis toxin for 16 hr reduced, in a concentration-dependent manner, the extent of [^{32}P]ADP-ribosylation of this 41,000-dalton protein (Fig. 1, lanes 2–7). Treatment of intact cyc$^-$ S49 cells with 48 ng/ml of pertussis toxin resulted in a 25% decrease in subsequent pertussis toxin-catalyzed [^{32}P]ADP-ribosylation, while treatment with 190 ng/ml of pertussis toxin resulted in a 96% decrease. This 41,000-dalton protein is presumably the alpha subunit of G_i, since the alpha subunit of G_i is approximately 41,000 daltons, and G_i is the only known pertussis toxin substrate in cyc$^-$ S49 cells.

As has been reported previously [9], somatostatin was able to inhibit forskolin-stimulated accumulation of cyclic AMP in intact wild-type S49 lymphoma cells (Fig. 2). Maximum inhibition (50–70%) was observed with 1 μM somatostatin. Treatment of wild-type S49 cells with 50–100 ng/ml of pertussis toxin completely blocked the capacity of somatostatin to inhibit the accumulation of cyclic AMP (Fig. 2).

β-Adrenergic receptors did not inhibit basal or forskolin-stimulated adenylate cyclase activity in membranes prepared from cyc$^-$ S49 lymphoma cells (data not shown). However, Gpp(NH)p and GTPγS inhibited forskolin-stimulated adenylate cyclase activity in membranes prepared from cyc$^-$ S49 cells (Fig. 3). Treatment of intact cyc$^-$ S49 cells with pertussis toxin did not alter significantly the ability of forskolin to stimulate the activity of adenylate

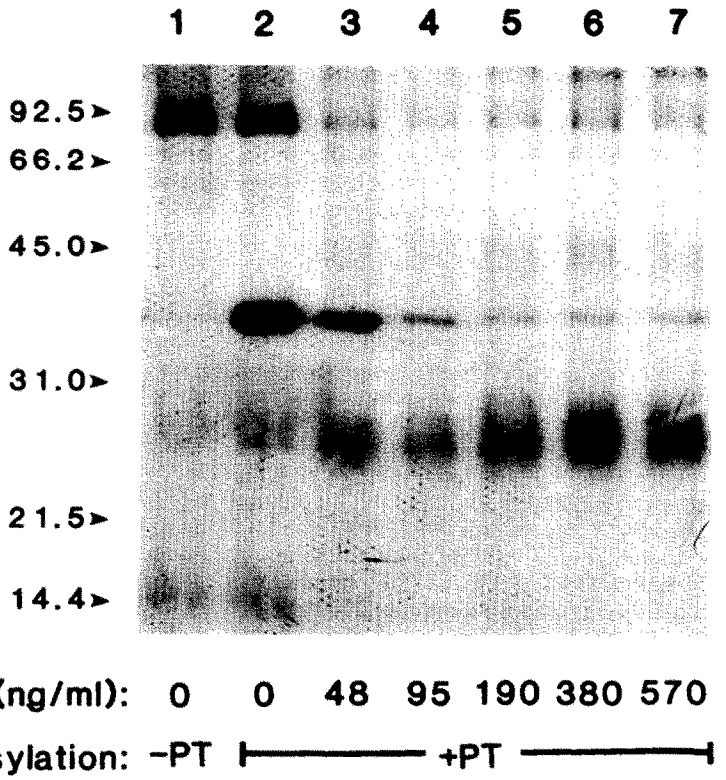

Fig. 1. Effect of treatment of cyc⁻ S49 lymphoma cells with pertussis toxin on *in vitro* [^{32}P]ADP-ribosylation of G_i by pertussis toxin. Cyc⁻ S49 lymphoma cells were treated for 16 hr with various concentrations of pertussis toxin. Membranes prepared from the cells were then incubated *in vitro* with [^{32}P]NAD in the presence or absence of pertussis toxin as described in Materials and Methods. The lanes represent: (1) membranes from control cells incubated *in vitro* in the absence of pertussis toxin; (2) membranes from control cells incubated *in vitro* with pertussis toxin; (3–7) membranes from cells pretreated with pertussis toxin (48, 95, 190, 380, and 570 ng/ml respectively) incubated *in vitro* with pertussis toxin. The 41,000-dalton protein labeled *in vitro* represented 24.7, 6.2, 3.7, 3.7 and 3.3% of control for lanes 3–7 respectively.

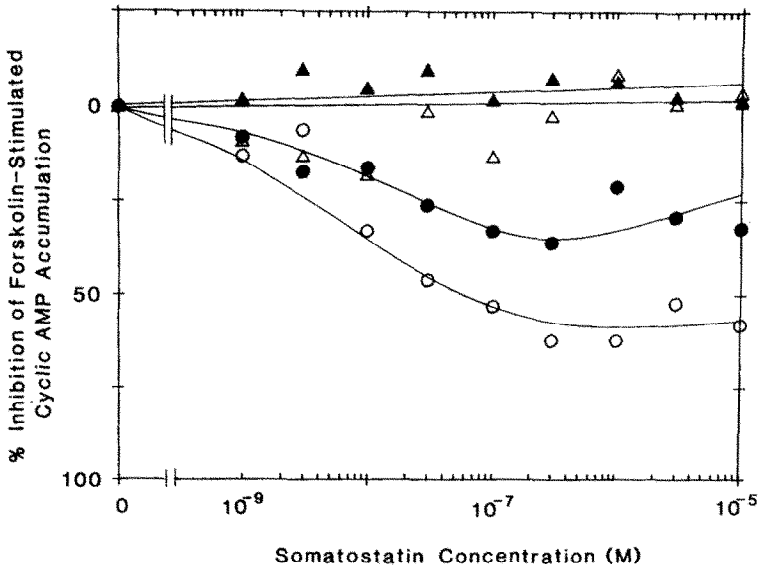

Fig. 2. Effect of treatment of wild-type S49 lymphoma cells with pertussis toxin on accumulation of intracellular cyclic AMP. Wild-type S49 lymphoma cells were treated for 16 hr without (○) or with 25 ng/ml (●), 50 ng/ml (△), or 100 ng/ml (▲) of pertussis toxin. Cells were collected by centrifugation at 2500 *g* for 10 min and were resuspended for determination of accumulation of intracellular cyclic AMP. All assays included 1.0 mM forskolin, and the data are expressed as percent inhibition of forskolin-stimulated conversion of [^{3}H]ATP to [^{3}H]cAMP versus the concentration of somatostatin.

cyclase in membranes prepared from these cells. However, greater concentrations of Gpp(NH)p and GTPγS were required to inhibit adenylate cyclase activity in membranes prepared from treated cells (Fig. 3). GTP and GTPγS also inhibited forskolin-stimulated adenylate cyclase activity in membranes prepared from L6 myoblasts. Inhibition by guanine nucleotides was prevented completely by prior treatment of these cells with pertussis toxin (data not shown).

Treatment of intact wild-type S49 lymphoma cells with pertussis toxin increased the basal activity of adenylate cyclase in membranes prepared from these cells. Isoproterenol-stimulated activity was increased by approximately 2-fold in membranes prepared from pertussis toxin-treated cells (Fig. 4A). The EC_{50} value of isoproterenol for stimulation of adenylate cyclase activity was not affected by treatment with pertussis toxin.

Treatment of intact L6 myoblasts with pertussis toxin did not significantly affect basal activity of adenylate cyclase in membranes prepared from these cells. However, the ability of isoproterenol to stimulate adenylate cyclase activity was increased by approximately 2-fold in membranes prepared from treated cells (Fig. 4B). The EC_{50} value of isoproterenol for stimulation of adenylate cyclase activity was not affected by treatment with pertussis toxin.

The properties of β-adrenergic receptors on membranes prepared from L6 myoblasts were assessed directly using the antagonist [^{125}I]IPIN and the agonist [^{3}H]HBI (Table 1). The affinity of β-adrenergic receptors on membranes prepared from L6 myoblasts for [^{125}I]IPIN and the density of receptors determined with [^{125}I]IPIN were unaffected by prior treatment of intact cells with pertussis toxin. Similarly, the affinity of the receptors for [^{3}H]HBI

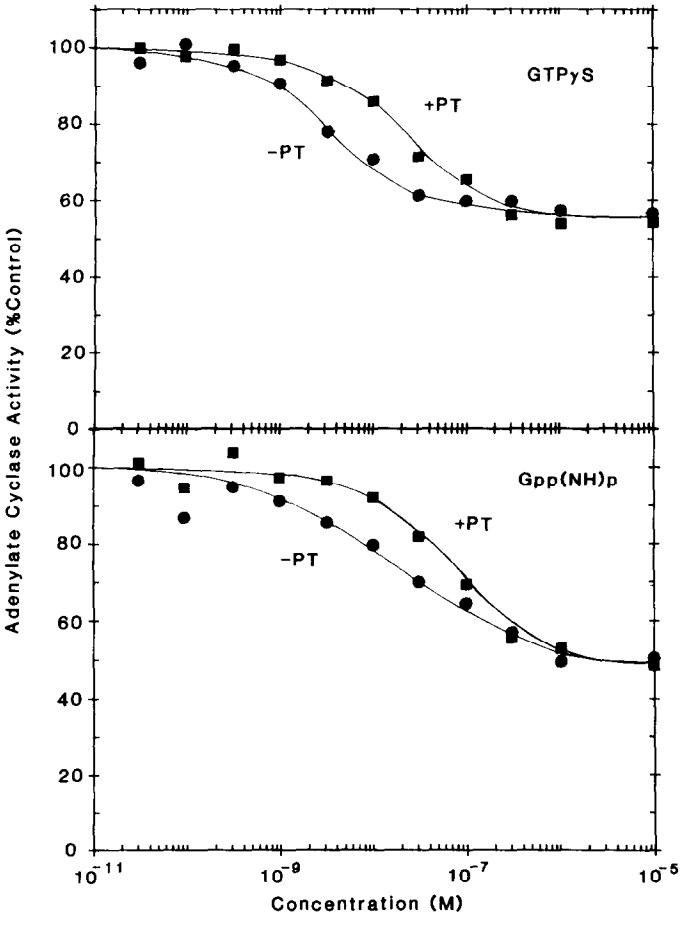

Fig. 3. Effect of treatment of cyc⁻ S49 lymphoma cells with pertussis toxin on inhibition of adenylate cyclase activity by guanine nucleotides. Cyc⁻ S49 lymphoma cells were treated for 16 hr with 190 ng/ml of pertussis toxin. Membranes prepared from control (●) or pertussis toxin-treated (■) cells (18.1 and 25.0 μg protein/assay respectively) were incubated with 100 μM forskolin and increasing concentrations of Gpp(NH)p or GTPγS. Data points are means of triplicate determinations and are presented as percent inhibition of activity in the presence of 100 μM forskolin (35 ± 1.0 pmol/mg of protein·min and 37 ± 2.1 pmol/mg of protein·min for control and treated cells respectively) versus the concentration (M) of nucleotide. Results are representative of two similar experiments.

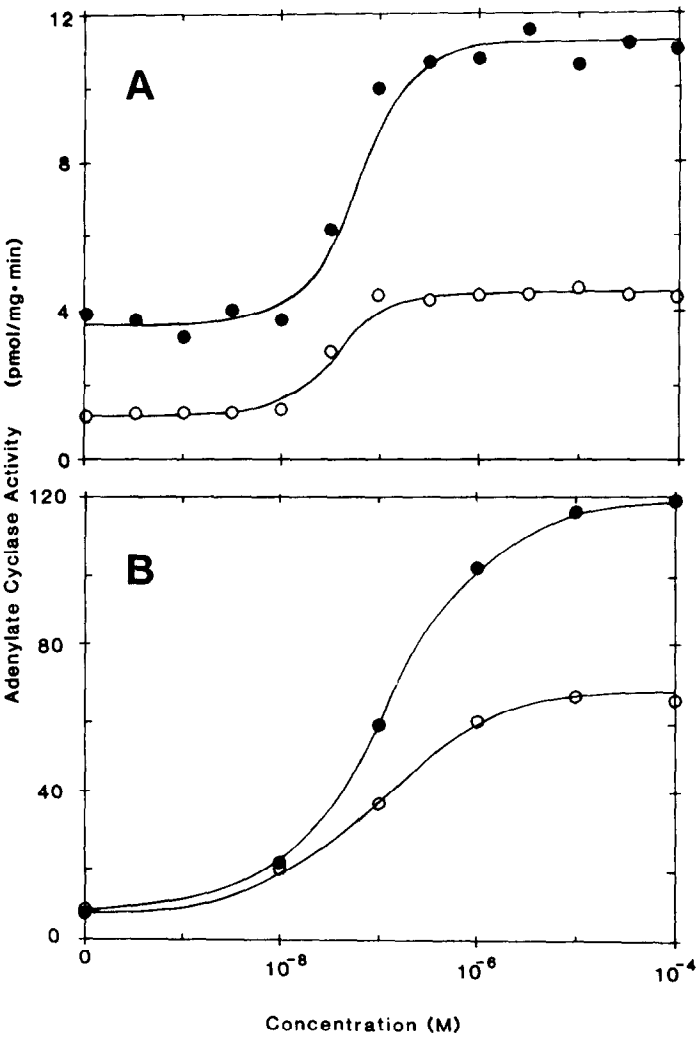

Fig. 4. Effect of treatment of wild-type S49 lymphoma cells and L6 myoblasts with pertussis toxin on stimulation of adenylate cyclase activity by isoproterenol. (A) Wild-type S49 lymphoma cells were treated with 190 ng/ml of pertussis toxin for 16 hr and membranes were prepared. (B) L6 myoblasts were treated with 200 ng/ml of pertussis toxin for 24 hr, and membranes were prepared as described in Materials and Methods. Adenylate cyclase activity (pmol/mg of protein · min) is plotted for control (○) and pertussis toxin-treated (●) cells versus the concentration (M) of isoproterenol. Data points are means of triplicate assays, and the results are representative of two similar experiments.

and the density of receptors determined with [^{3}H]HBI were unaffected by prior treatment of L6 myoblasts with pertussis toxin.

The properties of β-adrenergic receptors on membranes prepared from cyc$^-$ S49 lymphoma cells were also investigated. The affinity of β-adrenergic receptors on membranes prepared from cyc$^-$ S49 cells for [^{125}I]IPIN and the density of receptors determined with [^{125}I]IPIN were unaffected by prior treatment of intact cells with pertussis toxin (Table 1). However, the ability of β-adrenergic receptors to bind [^{3}H]HBI was reduced markedly after treatment of intact cyc$^-$ S49 cells with pertussis toxin (Fig. 5). These experiments required the treatment of a large volume of S49 cells with pertussis toxin (8–10 liters of cells, using up to 2.0 mg of pertussis toxin). Similar results were obtained in two separate experiments using two and three (Fig. 5) different concentrations of [^{3}H]HBI.

DISCUSSION

The ability of β-adrenergic receptors to interact with G_s is well established [27–29]. However, the guanine nucleotide-binding proteins G_s and G_i are similar in structure and function, and β-adrenergic receptors do not have an absolute specificity for G_s. For instance, when purified β-adrenergic receptors and G_i were reconstituted into artificial lipid vesicles, the addition of agonists resulted in an increase in the ability of G_i to bind GTPγS and hydrolyze GTP [29, 30]. Although the relative affinities of β-adrenergic receptors for G_s and G_i are not known, G_i is more abundant than G_s, and it is possible that β-adrenergic

Table 1. Properties of β-adrenergic receptors on membranes prepared from cyc⁻ S49 lymphoma cells and L6 myoblasts after treatment of cells with pertussis toxin

Cell type	Ligand	Condition	N	K_d (pM)	B_{max} (fmol/mg protein)
L6 Myoblasts	[^{125}I]IPIN	Control	4	16.7 ± 1.3	367 ± 7
		Treated	4	18.8 ± 1.1	338 ± 51
	[^{3}H]HBI	Control	3	403 ± 30	171 ± 30
		Treated	4	442 ± 32	162 ± 51
Cyc⁻ S49 Lymphoma cells	[^{125}I]IPIN	Control	8	18.0 ± 1.5	112 ± 5.6
		Treated	8	16.7 ± 2.1	99 ± 6.8

Cyc⁻ S49 lymphoma cells and L6 myoblasts were treated for 16 hr with 190 ng/ml of pertussis toxin, and membranes were then purified as described in Materials and Methods. The density (B_{max}) of β-adrenergic receptors and their affinity (K_d) for [^{125}I]IPIN and [^{3}H]HBI were determined in N separate experiments by Scatchard analysis of the saturation of receptors with eight concentrations of each radioligand. Values are means ± SEM.

receptors interact with G_i in intact cells. Unfortunately, the complete adenylate cyclase system found in normal cells is sufficiently complex that such an interaction may be difficult to detect. For this reason we chose to investigate the simpler system represented by cyc⁻ S49 lymphoma cells.

Although cyc⁻ S49 cells express β-adrenergic receptors [15] and G_i [9–11], they do not express messenger RNA coding for the alpha subunit of G_s [8], immunoreactive alpha subunit of G_s [8], or any of the functions of G_s [5–7]. Since G_i is the only known guanine nucleotide-binding protein in cyc⁻ S49 cells, possible interactions between β-adrenergic receptors and G_i can be investigated using membranes prepared from cyc⁻ S49 lymphoma cells. β-Adrenergic receptors on membranes prepared from cyc⁻ S49 cells have been shown to bind [^{3}H]HBI with high affinity, and the binding of [^{3}H]HBI is inhibited by GTP [15]. In addition, receptors labeled with [^{3}H]HBI appear to have a larger molecular size compared to receptors labeled with [^{125}I]IPIN [16]. These results are similar to those observed in experiments carried out with membranes prepared from wild-type S49 cells [15, 16], as well as with membranes prepared from other tissues [13, 14, 16]. In tissues that contain functional G_s, the high-affinity, GTP-sensitive binding of agonists and the larger apparent molecular size of agonist-bound receptors

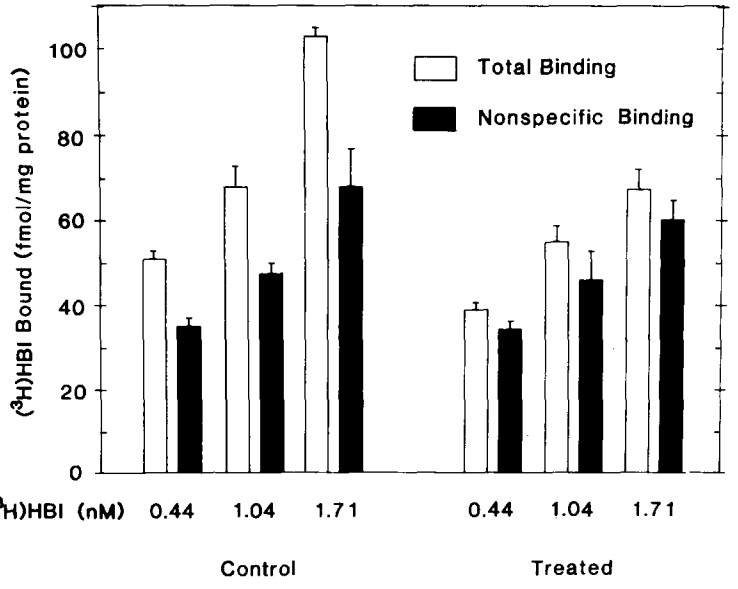

Fig. 5. Effect of treatment of cyc⁻ S49 lymphoma cells with pertussis toxin on the ability of β-adrenergic receptors to bind [^{3}H]HBI. Cyc⁻ S49 lymphoma cells were treated for 16 hr with (treated) or without (control) pertussis toxin (190 ng/ml), and membranes were purified as described in Materials and Methods. Membranes (489 and 619 μg protein/assay for control and treated cells respectively) were incubated with [^{3}H]HBI (0.44, 1.04, or 1.71 nM) in the presence (closed bars) or absence (open bars) of 1.0 μM propranolol. Assays were performed in triplicate, and the results are representative of two similar experiments.

are thought to reflect the formation of a ternary complex composed of agonist, receptor, and G_s [6, 13, 14]. However, since cyc$^-$ S49 cells do not express functional G_s, similar results obtained with these cells may reflect the formation of a ternary complex composed of agonist, receptor, and G_i [15, 16].

In this study, the possibility that β-adrenergic receptors interact with G_i was investigated using S49 lymphoma cells and L6 myoblasts treated with pertussis toxin. Several approaches were used to assess the functional activity of G_i after treatment of cells with pertussis toxin. In membranes from both cyc$^-$ S49 lymphoma cells and L6 myoblasts, GTP and analogues of GTP inhibited forskolin-stimulated adenylate cyclase activity. In addition, somatostatin was shown to inhibit forskolin-stimulated enzyme activity in intact wild-type S49 lymphoma cells. These effects were blocked or attenuated by treatment of intact cells with pertussis toxin and are therefore thought to be mediated by G_i.

Because inhibition of forskolin-stimulated adenylate cyclase activity by analogues of GTP in cyc$^-$ S49 cells was not blocked completely by pertussis toxin, the effect of pertussis toxin was also assessed by quantifying pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of membranes prepared from cyc$^-$ S49 cells. Although several membrane proteins incorporated radioactivity from [^{32}P]NAD in the absence of pertussis toxin, only the 41,000-dalton alpha subunit of G_i was specifically [^{32}P]ADP-ribosylated by pertussis toxin. Treatment of intact cyc$^-$ S49 cells with pertussis toxin caused almost complete loss of subsequent pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of G_i, indicating that G_i was almost completely ADP-ribosylated by endogenous NAD during the exposure of intact cells to the toxin.

Treatment of intact wild-type S49 cells with pertussis toxin resulted in an increase in the basal activity of adenylate cyclase. This suggests that tonic inhibitory activity of G_i, responsible for the low basal activity of adenylate cyclase in these cells, was lost after ADP-ribosylation of G_i by pertussis toxin. Failure of pertussis toxin to affect basal activity of adenylate cyclase in L6 cells suggests that G_i is not tonically active in these cells. Treatment of intact wild-type S49 cells and L6 myoblasts with pertussis toxin potentiated the ability of isoproterenol to stimulate adenylate cyclase activity, suggesting that G_i normally acts to attenuate hormone-stimulated adenylate cyclase activity. These observations reflect the complexity of systems that contain both G_s and G_i.

Two possible mechanisms exist whereby G_i could attenuate β-adrenergic receptor-stimulated adenylate cyclase activity. Since the beta subunit of G_i is capable of inhibiting activated G_s through interaction of the beta subunit of G_i with the alpha subunit of G_s [1–4], G_i could attenuate β-adrenergic receptor-stimulated adenylate cyclase activity by acting as a source, or reservoir, of beta subunits. Alternatively, β-adrenergic receptors could interact directly with G_i. Such an interaction could result in activation of G_i or in sequestration of β-adrenergic receptors away from G_s, ultimately resulting in an attenuated ability of receptors to stimulate the activity of adenylate cyclase.

Since β-adrenergic receptors did not inhibit basal or forskolin-stimulated adenylate cyclase activity, even in cyc$^-$ S49 cells, they apparently do not activate G_i. However, β-adrenergic receptors may interact directly with G_i to produce an inactive complex. To test the hypothesis that pertussis toxin-induced potentiation of isoproterenol-stimulated adenylate cyclase activity resulted from blocking the formation of an inactive complex between β-adrenergic receptors and G_i, the properties of receptors on membranes prepared from pertussis toxin-treated and control cells were investigated using the antagonist [^{125}I]IPIN and the agonist [^{3}H]HBI. The absence of an observable effect of pertussis toxin on the properties of β-adrenergic receptors on membranes prepared from L6 myoblasts may be a consequence of the presence of functional G_s in these cells. Thus, in the absence of GTP and in the presence of a large amount of G_s, β-adrenergic receptors may preferentially interact with G_s rather than G_i. Preliminary experiments designed to examine the effect of pertussis toxin on the binding of [^{3}H]HBI to membranes derived from wild-type S49 lymphoma cells were inconclusive.

The possibility that β-adrenergic receptors can interact with G_i in the absence of functional G_s was examined in cyc$^-$ S49 cells. Treatment of cyc$^-$ S49 cells with pertussis toxin markedly decreased the ability of β-adrenergic receptors to bind the agonist [^{3}H]HBI but had no effect on the ability of receptors to bind the antagonist [^{125}I]IPIN. These results suggest that, in the presence of an agonist, β-adrenergic receptors on membranes prepared from cyc$^-$ S49 cells interact with G_i to form a ternary complex composed of agonist, receptor, and G_i. Although G_i is the only known pertussis toxin substrate in cyc$^-$ S49 cells, it is possible that an unidentified pertussis toxin substrate mediates the effects of pertussis toxin on the high-affinity binding of [^{3}H]HBI.

β-Adrenergic receptors have been shown to interact functionally with G_i when the purified proteins are reconstituted into artificial lipid vesicles [29, 30]. Results presented here support the hypothesis that such an interaction may also occur in cyc$^-$ S49 lymphoma cells. However, the extent of this interaction may depend upon the relative affinity of the receptor for G_s and G_i, and upon the relative amounts of G_s and G_i. An interaction between β-adrenergic receptors and G_i may only occur in the absence of G_s. Alternatively, the ability to detect the formation of complexes involving receptors and G_i may be a function of the experimental conditions. For example, assays of adenylate cyclase activity are carried out in the presence of GTP. Under these conditions, β-adrenergic-receptor complexes with G_s do not accumulate, and activated receptors may be free to interact with G_i as well as with G_s, since G_i may be in molar excess relative to the concentration of G_s. However, radioligand binding assays using [^{3}H]HBI are carried out in the absence of GTP. In these assays, the agonist-occupied receptor may preferentially interact with G_s due to its greater relative affinity for this protein. Thus, pertussis toxin treatment of L6 myoblasts could potentiate β-receptor-stimulated adenylate cyclase activity (since GTP is present in this assay), but have no effect on the

binding of [^{3}H]HBI to receptors in membranes derived from these cells (since GTP is absent in this assay).

An interaction between β-adrenergic receptors and G_i could explain the observation that β-adrenergic receptors can, under some conditions, inhibit the activity of adenylate cyclase [19, 31]. An interaction between β-adrenergic receptors and G_i could also be involved in desensitization of adenylate cyclase after prolonged stimulation by agonists. A role for G_i has been proposed for desensitization of glucagon-stimulated adenylate cyclase activity in intact hepatocytes [32], and for desensitization of vasopressin-stimulated adenylate cyclase activity in cultured rabbit renal cortical collecting tubular cells [33]. G_i may also be required for maximal stimulation of adenylate cyclase activity by β-adrenergic receptors [34]. An interaction between β-adrenergic receptors and G_i may represent a mechanism through which stimulation of the activity of adenylate cyclase by β-adrenergic receptors can be regulated. It may also represent a mechanism through which β-adrenergic receptors can affect the activity of cyclic AMP-independent cellular processes possibly mediated by G_i.

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