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Use of (−)-[^{3}H]Dihydroalprenolol to Study *Beta* Adrenergic Receptor-Adenylate Cyclase Coupling in C6 Glioma Cells: Role of 5'-Guanylylimidodiphosphate

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SUMMARY

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(−)-[^{3}H]Dihydroalprenolol, a potent *beta* adrenergic antagonist, was used as a ligand to characterize the *beta* adrenergic receptors coupled with adenylate cyclase in C6 glioma cell membranes. Binding of (−)-[^{3}H]dihydroalprenolol was saturable with 0.47 ± 0.03 pmole/mg of protein (N = 4) and occurred on a single category of specific binding sites. C6 glioma cells contained approximately 10,000 sites/cell. The dissociation binding constant of (−)-[^{3}H]dihydroalprenolol was 5.7 ± 0.9 nM (N = 5), and its apparent inhibition constant for the (−)-isoproterenol-activated adenylate cyclase was 2.9 ± 0.5 nM (N = 4). The association of (−)-[^{3}H]dihydroalprenolol with its binding sites was very fast, less than 1 min at 30°, at a ligand concentration of 45 nM. Dissociation was a first-order reaction, with a rate constant of 0.35 min^{-1}. An association rate constant of 6×10^7 M^{-1} min^{-1} was computed. Activation of the adenylate cyclase by (−)-isoproterenol (0.1 mM) was instantaneous. After prior saturation of the binding sites with (−)-[^{3}H]dihydroalprenolol, activation of the adenylate cyclase by (−)-isoproterenol was slow and limited by the dissociation of (−)-[^{3}H]dihydroalprenolol from its binding sites. *Beta* adrenergic agonists competed for (−)-[^{3}H]dihydroalprenolol binding sites and activated the adenylate cyclase with an effectiveness typical of *beta*$_1$ adrenergic specificity, since the order of potency was (−)-isoproterenol > (−)-norepinephrine ≅ epinephrine. Whatever the agonist considered, there was a 3-fold difference between the agonist dissociation constants determined by analyzing the competitive displacement of (−)-[^{3}H]dihydroalprenolol from binding sites, and the apparent affinity ($K_{A\ app}$) for adenylate cyclase activation. This difference indicates a hyperbolic relationship between receptor occupancy and adenylate cyclase activation. Complete activation of the enzyme requires full occupation of the specific binding sites. *Beta* blocking agents (but not *alpha* blocking agents or neuroleptics) inhibited (−)-[^{3}H]dihydroalprenolol binding and the activated adenylate cyclase in the same order of potency. The ratio of the antagonist dissociation constants for binding (K_D) to their apparent inhibition constants ($K_{i\ app}$) for adenylate cyclase was about 2 for (−)-[^{3}H]dihydroalprenolol and all the other *beta* antagonists tested. Like adenylate cyclase activation, binding was stereospecific, since (+)-propranolol required concentrations about 30 times higher than the (−) isomer to inhibit binding or adenylate cyclase stimulation half-maximally. These equilibrium and

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rate study experiments suggest that (−)-[^{3}H]dihydroalprenolol binding sites have all the features expected of *beta* adrenergic receptors functionally coupled with the adenylate cyclase. The GTP analogue 5′-guanylylimidodiphosphate [Gpp(NH)p] stimulated the basal adenylate cyclase catalytic state but reduced the maximal velocity of the agonist-activated state. The effect of Gpp(NH)p on these two adenylate cyclase states was irreversible. Gpp(NH)p reduced the affinity of (−)-isoproterenol for the *beta* receptor but increased its apparent affinity for adenylate cyclase activation. Gpp(NH)p therefore increased the efficiency of coupling between receptor occupancy and adenylate cyclase stimulation. It had no effect on the binding of (−)-alprenolol to the *beta* receptor. The following working hypothesis is proposed to explain these results: by modifying the equilibrium between the different states of the *beta* receptor-adenylate cyclase system, Gpp(NH)p changes the characteristics of the *beta* receptor when the receptor is "coupled" with the adenylate cyclase as a result of its interaction with an agonist.

INTRODUCTION

Three labeled blocking agents—[^{3}H]-propranolol (1, 2), (−)-[^{3}H]dihydroalprenolol (3–7) and [^{125}I]hydroxybenzylpindolol (8–12) have been shown to interact with stereospecific binding sites on particulate cell fractions containing *beta* adrenergic-sensitive adenylate cyclase. This report sets out to show that C6 glioma cells contain (−)-[^{3}H]dihydroalprenolol binding sites with the same specificity profile as the *beta* receptor implicated in adenylate cyclase activation. Although binding specificity is an essential feature of receptors, it cannot be taken as definite evidence identifying binding sites with functional receptors. The best illustration of this point is the report by Birnbaumer and Pohl (13) that [^{125}I]glucagon activated the adenylate cyclase of hepatic membranes instantaneously whereas [^{125}I]glucagon binding measured under the same assay conditions did not reach equilibrium for 10 or 15 min. Their explanation (13) was that only a few "specific" binding sites were functionally coupled with the catalytic component of adenylate cyclase and could thus be called "receptors." The remaining "specific" binding sites were called "acceptors" (14). Rodbell *et al.* (15) observed a nonlinear relationship between the levels of the glucagon receptors occupied and adenylate cyclase activation, which resembled the relationship reported for vasopressin receptors (16). Occupation of a small fraction of binding sites therefore leads to activation of a greater fraction of adenylate cyclase. On the other hand, Brown *et al.* (10) reported that the occupation of a fraction of [^{125}I]hydroxybenzylpindolol binding sites by (−)-*beta* adrenergic agonists resulted in proportionally less adenylate cyclase activation. Nevertheless, complete activation of the enzyme required full occupation of the binding sites in all the systems studied (15, 16, 10). We therefore analyzed in detail the relationship between the interaction of (−)-[^{3}H]dihydroalprenolol or (−)-isoproterenol with (−)-[^{3}H]dihydroalprenolol binding sites, and adenylate cyclase activation or inhibition under equilibrium conditions and also during kinetic experiments.

Guanine nucleotides are important in modulating the response of adenylate cyclase systems to catecholamines (for reviews, see refs. 17 and 18) and other hormones (for review, see ref. 18). 5′-Guanylylimidodiphosphate a synthetic analogue of guanosine triphosphate, has been used extensively to analyze the nucleotide triphosphate action mechanism affecting these systems. One of the most obvious effects of Gpp(NH)p[1] on catecholamine systems is to potentiate the influence of agonists and to increase their apparent affinity for adenylate cyclase stimulation. Contradictory observations have been reported as to whether these effects are due to a change in *beta* receptor characteristics. On the one hand, Gpp(NH)p did not seem to affect the affinity of [^{125}I]hydroxybenzylpindolol (10), [^{3}H]propranolol (2), or (−)-isoproterenol (10) for the binding sites, but on the other hand, Maguire

[1] The abbreviations used are: Gpp(NH)p, 5′-guanylylimidodiphosphate; cAMP, adenosine cyclic 3′,5′-monophosphate.

et al. and Lefkowitz *et al.* reported very recently that Gpp(NH)p reduced the apparent affinities of *beta* agonists without modifying those of *beta* antagonists for *beta* receptors in glioma cells (19) or frog erythrocytes (20). Similarly, GTP was shown to lower the affinity of glucagon and angiotensin for their binding sites in hepatic (21) and adrenal cortex (22) membranes, respectively. At the same time GTP increased the apparent affinity of glucagon for the adenylate cyclase system (15). The present experiments show that in C6 glioma cell membranes Gpp(NH)p reduces the apparent affinity of agonist for the *beta* receptor but does not affect antagonist affinity. However, Gpp(NH)p increases the apparent affinity of *beta* agonists for the *beta*-sensitive adenylate cyclase system. We suggest that the change in agonist-receptor interaction should not be ascribed to the direct effect of Gpp(NH)p on the receptor, but is simply a consequence of the influence of this nucleotide on one of the adenylate cyclase activation steps that follow such interaction.

MATERIALS AND METHODS

Cloned C6 glioma cells were taken from glial tumors induced by repeated injections of *N*-nitrosomethylurea into Wistar rats (23, 24). Cells were seeded in Roux glass bottles at a concentration of 2×10^6 cells/bottle. They were grown at 37° in Ham's F-10 medium supplemented with 10% fetal calf serum (Gibco), 50 IU/ml of penicillin G, and 5 mg/ml of streptomycin sulfate. The medium was changed 3, 5, and 6 days after plating. The cells were harvested at confluence on the 7th day.

Preparation of particulate fractions. The culture medium was removed by washing three times with 0.9% NaCl at room temperature. The cells were scraped off with a rubber stick and suspended in 0.9% NaCl. They were centrifuged for 10 min at $300 \times g$, and the pellet was washed once more in 0.9% NaCl and resuspended in a cold hypotonic medium (25 mM Tris-HCl, pH 8, and 5 mM EDTA; usually 6×10^6 cells/ml). The cell suspension was then homogenized in a Potter-Elvehjem glass homogenizer with a Teflon pestle (10 strokes). The homogenate was centrifuged at $5000 \times g$ for 20 min at 0°. The pellet was resuspended in the same hypotonic medium (usually 10×10^6 cells/ml), rehomogenized (five strokes), filtered through a silk screen (150-μm pore diameter), and centrifuged at $5000 \times g$ for 30 min. The final pellet was suspended in the same buffer (10^6 cells/10 μl), filtered again through the silk screen, and used for binding and adenylate cyclase assays within 1–2 hr. No significant change in binding or adenylate cyclase characteristics was detectable during this period. Filtration through the silk screen removed the DNA filaments formed after homogenization.

Measurement of (−)-[^{3}H]dihydroalprenolol binding. The particulate fractions (30 μg of protein) were incubated at 30° in 50 μl containing 100 mM Tris-HCl (pH 8), 5 mM $MgSO_4$, 1 mM cAMP, 0.2 mM ATP, 0.2 mg/ml of creatine kinase, 20 mM phosphocreatine, 1 mM EDTA, and (−)-[^{3}H]dihydroalprenolol. The (−)-[^{3}H]dihydroalprenolol concentration was calculated on the basis of the radioactivity added to the assay and the specific radioactivity of the product (32.6 Ci/mmole). Except in the kinetic experiments, the incubation medium was diluted 10 min later by adding 1 ml of cold (4°) washing medium containing 50 mM Tris-HCl (pH 8) and 20 mM $MgCl_2$. The diluted mixture was then carefully sprayed over two Whatman GF/C glass fiber filters, which were rinsed with 4 ml of washing medium just before filtration. As soon as all the diluted mixture had been filtered, the filters were rinsed three times with 4 ml of washing medium. The entire dilution and filtration processes did not take more than 25 sec. The upper filter was then dried at 50° for 30 min and counted in Unisolve (8 ml). The use of two glass fiber filters reduced the blank value without affecting specific or nonspecific binding. Specific binding was defined as the difference between the amount of (−)-[^{3}H]dihydroalprenolol bound in the absence and presence of either 10 μM unlabeled (−)-alprenolol or (−)-isoproterenol. Specific and nonspecific binding were determined in triplicate and duplicate, respectively. The reproducibil-

ity of replicate samples was ±5%. Except where otherwise stated, binding values refer to specific binding. Nonspecific binding in the presence of (−)-isoproterenol was slightly higher than in the presence of (−)-alprenolol (see Fig. 4C). For routine experiments nonspecific binding was determined using (−)-alprenolol. Both specific and nonspecific binding were proportional to the protein concentration. Boiling the membranes eliminated specific but not nonspecific binding.

Adenylate cyclase assay. The same reaction mixtures were used to determine [^{3}H]dihydroalprenolol binding and adenylate cyclase activity, except that in the latter case trace amounts of [^{3}H]cAMP and [α-^{32}P]ATP were added (0.002 and 2 μCi/tube, respectively).

The reaction was started at zero time by adding membranes to all the other compounds except for the trace amount of [α-^{32}P]ATP, which was added after 9 min. The reaction was stopped 2 min later by addition of 900 μl of 5.5 mM Tris-HCl (pH 7.6), 0.33 mM ATP, 0.55 mM cAMP, and 10 mM $CaCl_2$. The tubes were centrifuged at 5000 × g for 5 min. [^{32}P]cAMP and [^{3}H]-cAMP were isolated according to Salomon *et al.* (25). This adenylate cyclase assay protocol was designed to measure the velocity of the reaction between 9 and 11 min after it had started and to make possible direct comparison with the binding determinations, which were made 10 min after the beginning of the reaction. It was possible to measure adenylate cyclase activity in the presence of (−)-[^{3}H]dihydroalprenolol, since this product adheres tightly to the Dowex column and does not interfere with [^{3}H]cAMP recovery.

The formation of cAMP in the absence and presence of (−)-isoproterenol was a linear function of time.

Determination of equilibrium constants for binding and adenylate cyclase activation and inhibition. K_D* is the dissociation constant of (−)-[^{3}H]dihydroalprenolol calculated from the Scatchard plot. K_D refers to the dissociation constant of unlabeled agonists and antagonists for the (−)-[^{3}H]dihydroalprenolol binding sites and was calculated from the concentrations of these ligands that caused 50% inhibition of binding. Thus

$$[I_{50}] = K_D\left(1 + \frac{[S^*]}{K_D^*}\right)$$

where [S*] is the (−)-[^{3}H]dihydroalprenolol concentration.

$K_{A\ app}$ refers to the agonist activation constant for the adenylate cyclase system and is equal to the agonist concentration yielding 50% of maximal activation. $K_{i\ app}$ refers to the antagonist inhibition constant for (−)-isoproterenol-activated adenylate cyclase and was evaluated in two ways. (a) According to the increase in $K_{A\ app}$ for (−)-isoproterenol in the presence of a constant antagonist concentration [I], so that

$$K_{A\,app_1} = K_{A\,app}\left(1 + \frac{[I]}{K_{i\,app}}\right)$$

where $K_{A\ app_1} = K_{A\ app}$ in the presence of the antagonist. (b) According to the extent of inhibition of (−)-isoproterenol-activated adenylate cyclase caused by increasing antagonist concentrations, where

$$[I_{50}] = K_{i\,app}\left(1 + \frac{[S]}{K_{A\,app}}\right)$$

and [S] is the (−)-isoproterenol concentration.

Materials. ATP (disodium salt), (−)-isoproterenol, (−)-norepinephrine, (−)-epinephrine, dichloroisoproterenol, and (±)-propranolol were purchased from Sigma Chemical Company. cAMP, Gpp(NH)p, phosphocreatine, and creatine kinase were obtained from Boehringer/Mannheim. Calbiochem was the source of dopamine. Drugs were kindly donated as follows: phentolamine and unlabeled (−)-alprenolol, Ciba-Geigy Laboratories; phenoxybenzamine, Smith Klein & French Laboratories; pindolol, Sandoz; (−)- and (+)-propranolol, ICI-Pharma; fluphenazine, Squibb; chlorpromazine, Rhone Poulenc; and protokylol, Lakeside Laboratories. Hydroxybenzylpindolol was kindly donated by Dr. H. Glossmann, and ubiquitin, by Dr. G. Goldstein.

[^{3}H]cAMP (ammonium salt); 25 Ci/mmole), [α-^{32}P]ATP (sodium salt; 10–20 Ci/mmole), and (−)-[^{3}H]dihydroalprenolol (32.6 Ci/mmole) were purchased from New England Nuclear.

RESULTS

Kinetics of (−)-[^{3}H]dihydroalprenolol binding. The forward binding rate was very fast (Fig. 1). In the presence of 44 nM ligand, maximal binding was achieved within the first minute and, after a slight overshoot, remained stable for up to 20 min (Fig. 1). When a large excess of unlabeled ligand (0.1 mM) was added during the assay, binding was completely reversible within 10 min. Dissociation followed first-order kinetics; the semilogarithmic binding reversibility plot had a single slope that permitted calculation of a dissociation rate constant (k_{-1}) of 0.35 min^{-1}.

Kinetic analysis of relationship between the occupation of (−)-[^{3}H]dihydroalprenolol binding sites by (−)-[^{3}H]dihydroalprenolol or (−)-isoproterenol and adenylate cyclase activation. When 90% of the binding sites were occupied by the labeled ligand [in the presence of 50 nM (−)-[^{3}H]dihydroalprenolol], the addition of a large excess of (−)-isoproterenol (0.1 mM) made it possible to detect the dissociation of the labeled ligand, which followed a kinetic pathway very close to that observed after the addition of (−)-alprenolol (0.1 mM) (Fig. 2B). On account of the large excess of (−)-isoproterenol (0.1 mM) added, the (−)-[^{3}H]dihydroalprenolol binding reversal rate also indicates the forward rate of (−)-isoproterenol binding to the (−)-[^{3}H]dihydroalprenolol sites. In an experiment performed simultaneously using the same protocol, kinetic analysis of adenylate cyclase activation was performed by measuring the reaction velocity during 1-min periods for up to 10 min after the addition of (−)-isoproterenol (Fig. 2A). Under these conditions, adenylate cyclase activation was time-dependent and followed the same kinetic course as (−)-isoproterenol interaction with the binding sites, assessed under identical conditions (Fig. 2B). In contrast, when the adenylate

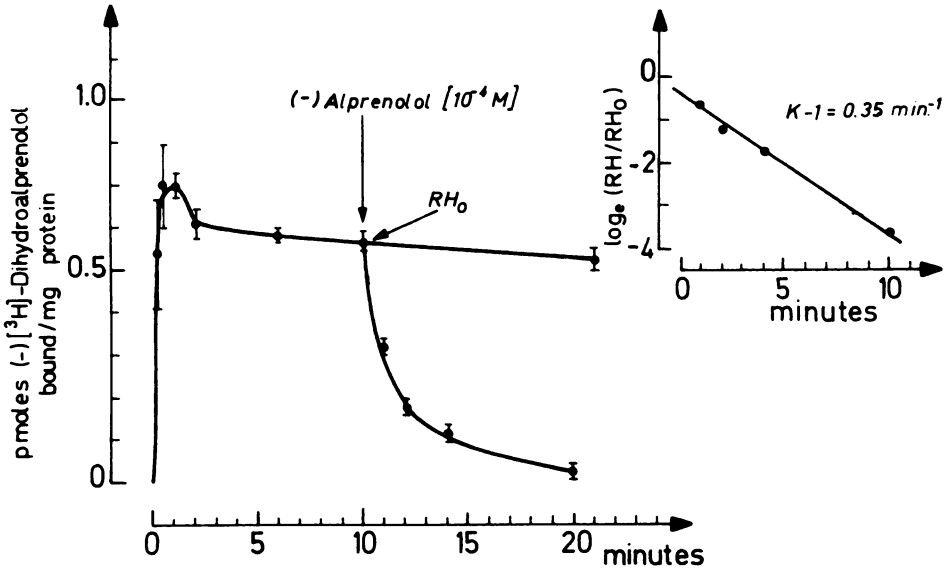

FIG. 1. *Time course for (−)-[^{3}H]dihydroalprenolol association with and dissociation from C6 glioma cell membranes*

C6 glioma cell membranes (29 μg of protein) were incubated with 44 nM; (−)-[^{3}H]dihydroalprenolol at 30°C. Each point is the mean ± standard error of three determinations. Inset: Semilogarithmic plot of the dissociation process. RH = concentration of the hormone-receptor complex; RHo = concentration of the hormone-receptor complex when the dissociation is started.

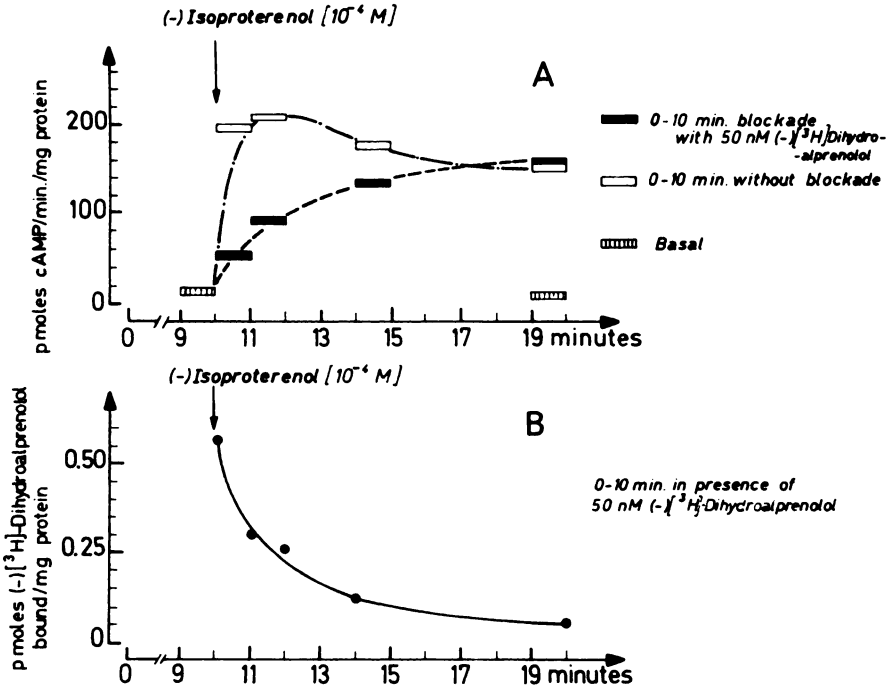

FIG. 2. *Comparison between rate of adenylate cyclase activation by (−)-isoproterenol and its interaction with (−)-[³H]dihydroalprenolol binding sites*

A. Time course for adenylate cyclase activation with or without a preliminary blockade of binding sites. Membrane (29 μg) were incubated in 40 μl of medium with and without 50 nM (−)-[³H]dihydroalprenolol. Ten minutes later, 5 μl of a (−)-isoproterenol solution (final concentration, (0.1 mM) were added and at various intervals thereafter labeled ATP was added in 5 μl of solution. The reaction was allowed to proceed for 1 min, as indicated by the length of the bars. Hatched bars indicated basal adenylate cyclase activity.

B. Time course for association of (−)-isoproterenol (0.1 mM) after preliminary occupation of the binding sites with (−)-[³H]dihydroalprenolol (50 nM). Experimental conditions were identical with those described in part A.

cyclase activation kinetic pathway was followed without a preliminary blockade, the action of (−)-isoproterenol (0.1 mM) was instantaneous (Fig. 2A). These experiments indicate that the rate-limiting step in adenylate cyclase activation by (−)-isoproterenol is its interaction with the (−)-[³H]dihydroalprenolol binding sites.

Adenylate cyclase activation by 500 nM (−)-isoproterenol was also instantaneous (Fig. 3). Blockade of the activation by (−)-[³H]dihydroalprenolol (200 nM) was complete within 1 min. In an experiment performed simultaneously, the binding kinetics of (−)-[³H]dihydroalprenolol (200 nM) was studied after a 6-min preliminary incubation with 500 nM (−)-isoproterenol. At 1, 2, 3, and 5 min after addition of (−)-[³H]dihydroalprenolol, the binding values were 0.55, 0.55, 0.62, and 0.60 pmole/mg of protein, respectively.

Affinities of (−)-[³H]dihydroalprenolol binding sites and adenylate cyclase system for labeled and unlabeled (−)-alprenolol and (−)-isoproterenol, and determination of (−)-[³H]dihydroalprenolol binding site density in C6 glioma cells. The same membrane preparation and the same stock solutions for labeled and unlabeled products were used for all the experiments reported in the present section. Specific (−)-[³H]dihydroalprenolol binding was a saturable process as a function of (−)-[³H]dihydroalprenolol concentration (Fig. 4A). In contrast, nonspecific binding, as defined under MATERIALS AND METHODS, increased linearly. Nonspecific binding therefore represented 10% of specific bind-

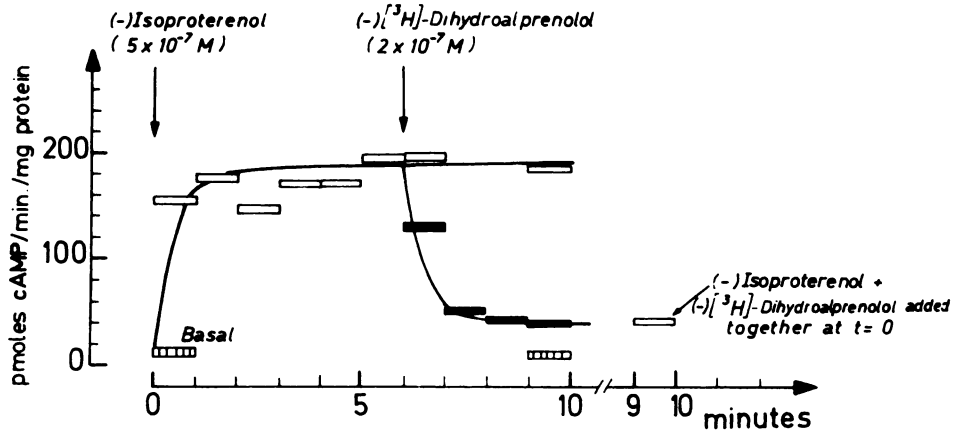

FIG. 3. *Time course for adenylate cyclase activation by (−)-isoproterenol and subsequent inhibition by (−)-[³H]hydroalprenolol*

Membranes (34 μg of protein in 10 μl) were added at zero time in test tubes containing 30 μl (□) or 35 μl (■) of incubation medium equilibrated at 30° and containing 500 nM (−)-isoproterenol. During the course of this experiment, adenylate cyclase activity was measured by adding labeled ATP (5 μl) and allowing the reaction to proceed for 1 min (length of the bars). Hatched bars represent basal adenylate cyclase activity. (−)-[³H]Dihydroalprenolol [200 nM made up with 50 nM (−)-[³H]dihydroalprenolol plus 150 nM unlabeled alprenolol] was added at 6 min, and the corresponding adenylate cyclase activities are represented by the solid bars.

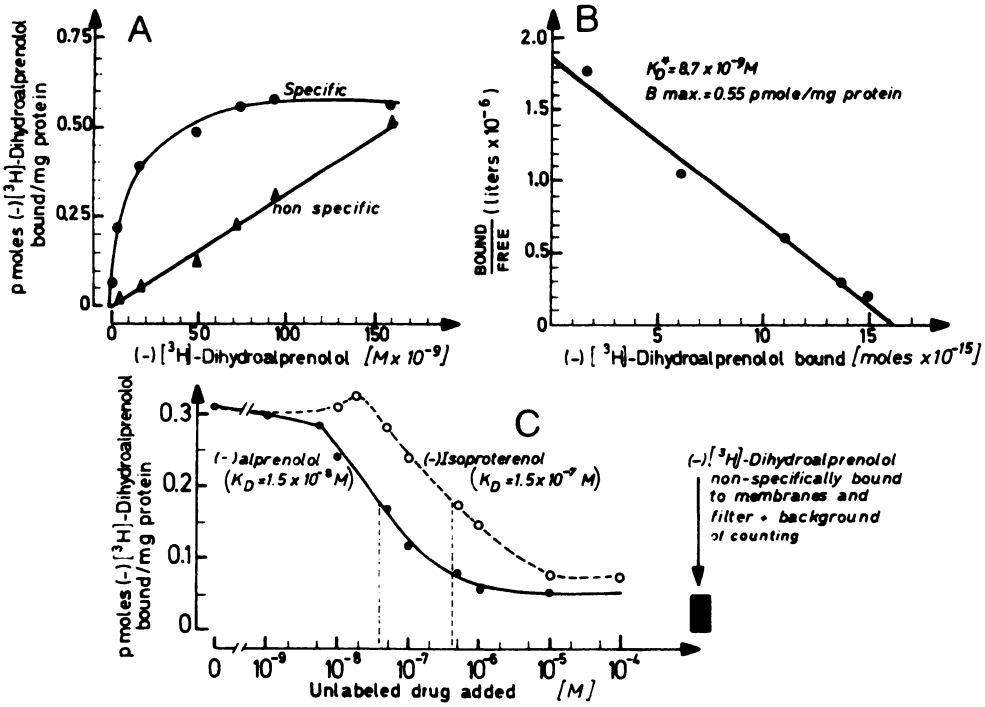

FIG. 4. *Affinities of (−)-[³H]dihydroalprenolol binding sites for (−)-alprenolol and (−)-isoproterenol, and determination of number of binding sites*

The membrane concentration was 27 μg of protein in 50 μl of solution. A. Dependence of (−)-alprenolol specific and nonspecific binding to C6 glioma cell membranes on the concentration of (−)-[³H]dihydroalprenolol. B. Scatchard plot of the data for specific binding. C. Competition between (−)-[³H]dihydroalprenolol and (−)-alprenolol or (−)-isoproterenol for the binding sites. The (−)-[³H]dihydroalprenolol concentration was 13.6 nM.

ing at 10 nM, the concentration generally used, and was equal to specific binding at 150 nM. The Scatchard plot of the dose-binding curve produced only one straight line, allowing determination of a dissociation constant (K_D*) of 8.7 nM in this experiment (Fig. 4B). The mean value of five experiments was 5.7 ± 0.9 nM (SEM). The total binding capacity of the membrane fraction used was 0.55 pmole/mg of protein (0.47 ± 0.03 pmole/mg; N = 5). On the basis of the total number of glioma cells used and the maximal binding capacity of the membrane, it may be calculated that the intact cells contained 7000 receptors/cell, assuming that no binding was lost during cell preparation. The exact loss of binding sites cannot be estimated, since the Scatchard plot of the binding curve obtained with homogenates was not linear. If recovery of the (−)-isoproterenol-sensitive adenylate cyclase (71%) is considered equal to the recovery of binding sites, the intact cells would contain 9860 receptors/cell.

As shown in Table 1, neuroblastoma cells did not contain either (−)-isoproterenol-sensitive adenylate cyclase or specific (−)-[^{3}H]dihydroalprenolol binding sites.

The apparent inhibition constant of (−)-[^{3}H]dihydroalprenolol for activated adenylate cyclase inhibition was $K_{i\,app}$ = 4.3 nM (Fig. 5A) (2.9 ± 0.5 nM; N = 4). The K_D of unlabeled (−)-alprenolol was 15 nM (Fig.

TABLE 1

Cell specificity of beta-sensitive adenylate cyclase and (−)-[^{3}H]dihydroalprenolol binding sites

The membrane concentrations of neuroblastoma and C6 glioma cells were 19 and 52 μg of protein, respectively, in 50 μl of solution.

Cells	Adenylate cyclase activity			(−)-[^{3}H]Dihydroalprenolol binding	
	Basal	+(−)-Isoproterenol (0.1 mM)	+PGE_1 (5 μM)	Nonspecific	Specific
	pmoles/2 min/mg protein			*pmoles/mg protein*	
Neuroblastoma	10.1	10.5	55.3	0.38	0.00
C6 glioma	6.1	95.6	11.3	0.06	0.36

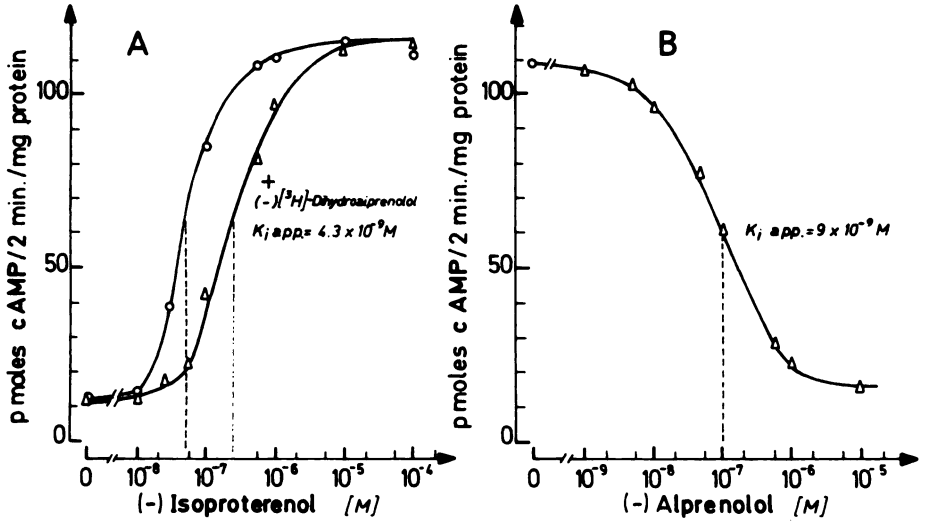

FIG. 5. *Determination of (−)-alprenolol inhibition constant ($K_{i\,app}$) for (−)-isoproterenol-activated adenylate cyclase*

Membrane preparation and drug solutions used were the same as in Fig. 4. A. Dose-response curve for adenylate cyclase activation by (−)-isoproterenol in the presence and absence of 13.6 nM (−)-[^{3}H]dihydroalprenolol. B. Dose-dependent inhibition of (−)-isoproterenol-activated adenylate cyclase by (−)-alprenolol. The (−)-isoproterenol concentration was 500 nM.

4C) (15 ± 4 nM; $N = 5$), and its $K_{i\ app}$ was 9 nM (Fig. 5B) (7.6 ± 1.2 nM; $N = 5$). The $K_D/K_{i\ app}$ ratios were 2 and 1.7 for (−)-[^{3}H]dihydroalprenolol and (−)-alprenolol, respectively. The difference between the absolute values of the constants (K_D and $K_{i\ app}$) for labeled and unlabeled ligand was due to the fact that, whereas the exact concentration of the labeled product was known (see MATERIALS AND METHODS), the concentration of unlabeled product had to be calculated according to the dilution process and was consequently overestimated because of (−)-alprenolol adsorption to plastic and glass walls.

The dissociation constant of (−)-isoproterenol for the binding sites was 150 nM (Fig. 4C) (180 ± 40 nM; $N = 8$), and its apparent activation constant ($K_{A\ app}$) was 50 nM (Fig. 5A) (62 ± 17 nM; $N = 5$). The ratio $K_D/K_{A\ app}$ was therefore 3. In all binding experiments, a slight increase in binding capacity was observed when low concentrations of (−)-isoproterenol were added (see, for example, Figs. 4C, 6, and 9C). This increase were never observed for antagonists.

Structure-activity relationships. All compounds which were able either to stimulate the basal adenylate cyclase or to inhibit the (−)-isoproterenol-activated adenylate cyclase were also competitors for (−)-[^{3}H]dihydroalprenolol binding (Figs. 6 and 7). The binding ratios of K_D to either $K_{A\ app}$, relating to agonists, or $K_{i\ app}$, relating to antagonists, were constant whatever the drug considered, and equal to 3 and 1.7 for *beta* adrenergic agonists and antagonists, respectively (Figs. 6 and 7, insets). Binding sites and the adenylate cyclase activation process were stereospecific, since (−)-propranolol was 27 and 36 times more potent than (+)-propranolol for binding and adenylate cyclase inhibition, respectively (Fig. 7, inset). Phentolamine, phenoxybenzamine, fluphenazine, and

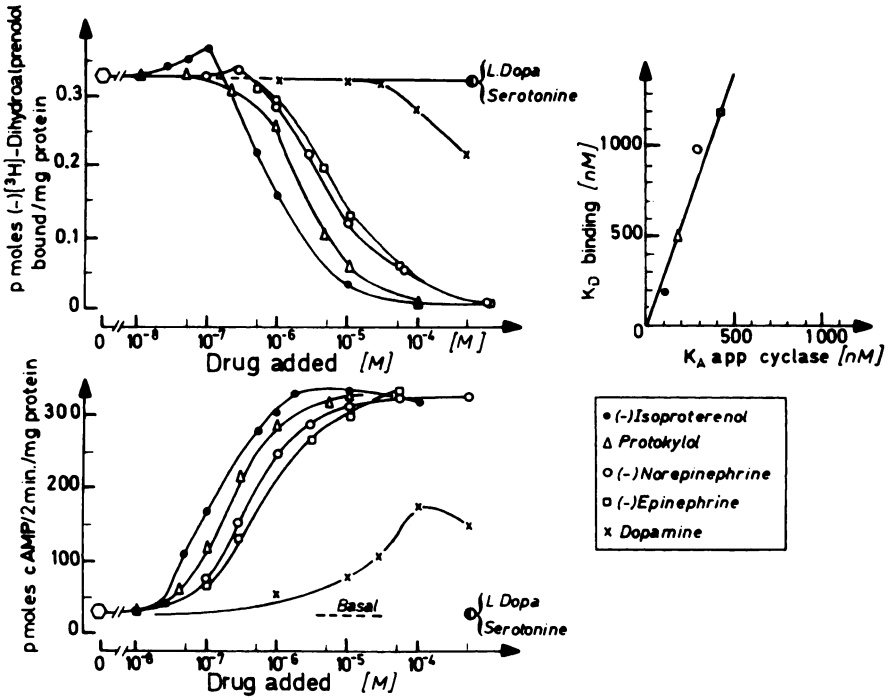

FIG. 6. *Comparison of relative potencies of various drugs in competing for binding sites and activating adenylate cyclase*

The membrane concentration was 50 μg of protein in 50 μl of solution. The (−)-[^{3}H]dihydroalprenolol concentration was 11.6 nM. The K_D* for (−)-[^{3}H]dihydroalprenolol, determined in the same experiment, was 3.3 nM, a value which was used to calculate the K_D of the various drugs for binding. Inset: Data plotted as a correlation between K_D for binding and $K_{A\ app}$ for adenylate cyclase activation.

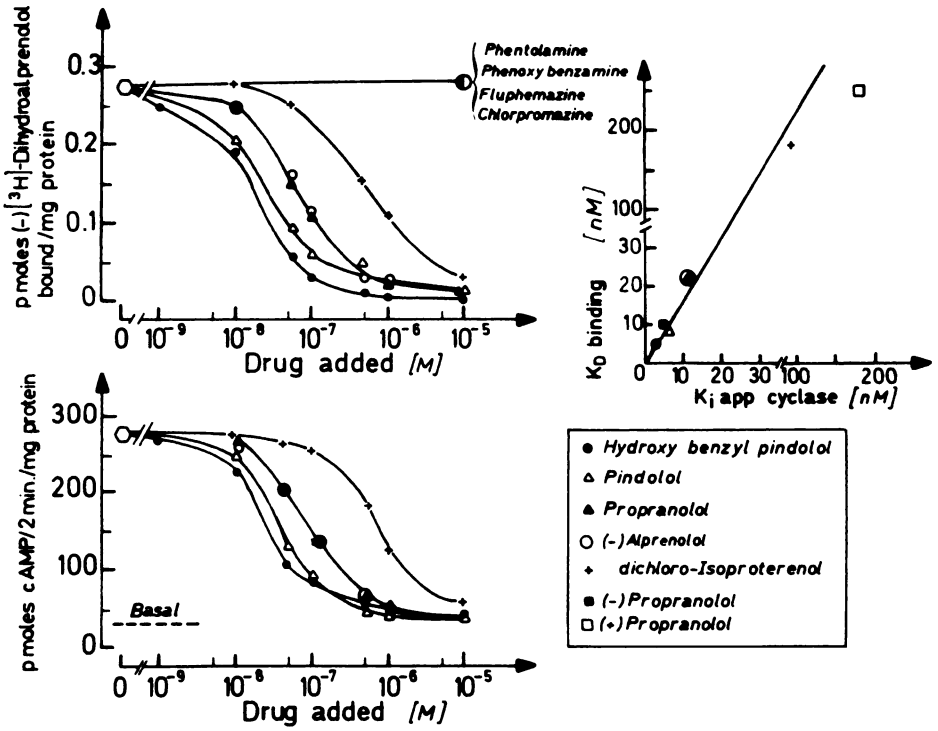

FIG. 7. *Comparison of relative potencies of various drugs in competing for binding sites and inhibiting (−)-isoproterenol-activated adenylate cyclase*

The membrane concentration was 42 μg of protein in 50 μl of solution. The (−)-[^{3}H]dihydroalprenolol concentration was 10 nM. The mean value of K_D* obtained in all other experiments (5.7 ± 0.9 nM; N = 5) was used to calculate K_D values for the unlabeled drugs. The (−)-isoproterenol concentration was 500 nM. The $K_{A\,app}$ for (−)-isoproterenol was taken equal to 50 nM in order to calculate the $K_{i\,app}$ value for the drugs. Inset: Data recorded in the left-hand part of the figure and those obtained in another experiment [for (−)- and (+)-propranolol] plotted as a correlation between K_D for binding and $K_{i\,app}$ for adenylate cyclase inhibition.

chlorpromazine were ineffective both as competitors for the binding sites and as inhibitors of (−)-isoproterenol-activated adenylate cyclase (Fig. 7). All the other neuroleptics tested at 10 μM (flupenthixol, thioproperazine, thioridazine, clozapine, haloperidol, and pimozide), as well as ubiquitin, were also ineffective (data not shown). L-Dopa and serotonin did not affect either binding or adenylate cyclase stimulation (Fig. 6).

Comparative effects of Gpp(NH)p on adenylate cyclase system and on interaction of beta adrenergic agonists and antagonists with (−)-[^{3}H]dihydroalprenolol binding sites. As in many other adenylate cyclase systems (for reviews, see refs. 17 and 18), Gpp(NH)p had a marked effect on C6 glioma cell adenylate cyclase. It stimulated the basal adenylate cyclase with a lag time of several minutes (Fig. 8B). However, it is the first system in which Gpp(NH)p has been shown to reduce the velocity of the reaction measured in the presence of a maximal concentration of agonist (Fig. 8A and B). Gpp(NH)p acted faster in the presence of (−)-isoproterenol than in its absence (Fig. 8B). Furthermore, when the velocity of the reaction was measured between 9 and 11 min after the addition of Gpp(NH)p, the dose-response curves for activation of the basal activity and inhibition of the (−)-isoproterenol-activated adenylate cyclase showed different apparent A_{50} values [concentration of Gpp(NH)p yielding half the maximal effect]. On the one hand, the dose-response curve for inhibition of (−)-

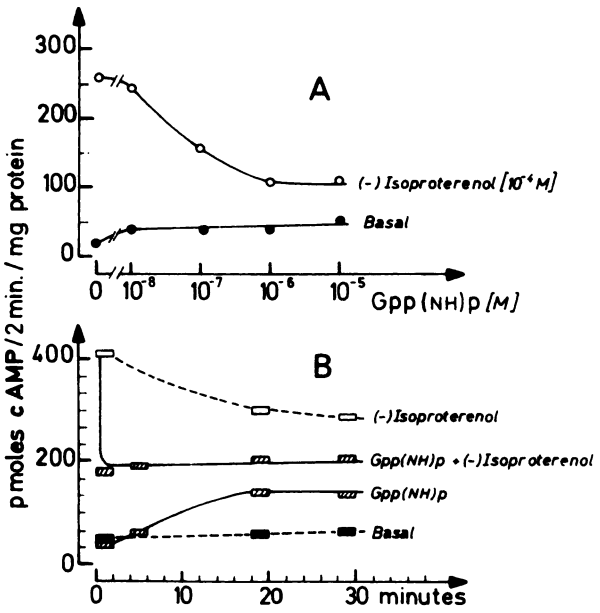

FIG. 8. *Gpp(NH)p effects on C6 glioma cell adenylate cyclase system*

The membrane concentration used was 35 μg of protein in 50 μl of solution. A. Dose dependence. Gpp(NH)p was added at zero time, and the adenylate cyclase activity was measured between 9 and 11 min according to the general protocol described under MATERIALS AND METHODS. B. Kinetics of Gpp(NH)p action on basal and (−)-isoproterenol-activated adenylate cyclase. After various preliminary incubation times, labeled ATP was added in 5 μl of solution, and the reaction was allowed to proceed for 2 min (length of the bars). The (−)-isoproterenol and Gpp(NH)p concentrations were 100 and 10 μM, respectively.

isoproterenol activation by Gpp(NH)p was probably measured under equilibrium conditions, since the lag time of the Gpp(NH)p effect in the presence of (−)-isoproterenol was very short (Fig. 8B). On the other hand, the effect of Gpp(NH)p on basal adenylate cyclase activities was probably not determined under equilibrium conditions for all Gpp(NH)p concentrations. If the lag times increased in inverse proportion to Gpp(NH)p concentration, we would expect an even lower A_{50} for basal stimulation at equilibrium than the one we observed (Fig. 8B). However, in pig kidney plasma membranes, Roy (26) has shown that the lag time for Gpp(NH)p activation was lower at 0.1 μM than at 10 μM. The situation is probably more complex, since free Gpp(NH)p (1 μM) was shown to be completely degraded after 10 min of incubation with hepatic membranes (27). Obviously, further investigations are needed to explain this difference in apparent A_{50}. As shown in Table 2, the action of Gpp(NH)p on either the basal or the (−)-isoproterenol-activated adenylate cyclase was irreversible, whereas the action of (−)-isoproterenol on basal adenylate cyclase was reversible.

A maximal dose of Gpp(NH)p (10 μM) increased the K_D of (−)-isoproterenol for the binding sites, from 120 to 380 nM (Fig. 9A), without modifying the K_D of (−)-alprenolol (Fig. 9B). Furthermore, Gpp(NH)p reduced the apparent activation constant ($K_{A\ app}$) for (−)-isoproterenol from 50 to 34 nM (Fig. 9C). In contrast, the nucleotide increased the apparent inhibition constant ($K_{i\ app}$) of (−)-alprenolol for the (−)-isoproterenol-activated adenylate cyclase (Fig. 9D). Thus the ratio of the K_D of (−)-isoproterenol for the binding sites to $K_{A\ app}$ for adenylate cyclase stimulation was 2.4 in the absence of Gpp(NH)p and 11 in its presence. Several similar experiments were conducted (Table 3). The presence of ATP did not change the amplitude of the shift in agonist affinity induced by Gpp(NH)p (Table 3).

The quantitative relationship between

TABLE 2

Reversibility of Gpp(NH)p and (−)-isoproterenol effects on adenylate cyclase system

The membranes, containing 80 μg of protein in 50 μl of solution, were first incubated under standard conditions with drugs added as shown below. After 10 min, 3 ml of cold (4°) standard medium (see MATERIALS AND METHODS) were added. (−)-Isoproterenol and Gpp(NH)p concentrations were 0.1 mM and 10 μM respectively. The suspensions were centrifuged at 5000 × *g* for 15 min at 4°. The supernatant was discarded, and the tubes were dried by aspiration. The pellets were resuspended in 45 μl of standard incubation medium, supplemented with drugs as indicated, and incubated at 30°. After 9 min, 5 μl of labeled ATP were added, and the reaction was allowed to proceed for 2 min.

Preliminary incubation	Adenylate cyclase assay	Cyclic AMP formed
		pmoles/2 min/mg protein
No additions	No additions	4.0
Gpp(NH)p	No additions	13.2
Gpp(NH)p	Gpp(NH)p	16.9
(−)-Isoproterenol	No additions	11.3
No additions	(−)-Isoproterenol	56.1
(−)-Isoproterenol	(−)-Isoproterenol	47.5
(−)-Isoproterenol + Gpp(NH)p	No additions	36.8
No additions	(−)-Isoproterenol + Gpp(NH)p	37.6
(−)-Isoproterenol + Gpp(NH)p	(−)-Isoproterenol + Gpp(NH)p	41.5

receptor occupancy and adenylate cyclase activation in the presence or absence of Gpp(NH)p was calculated and is reported in Fig. 10.

DISCUSSION

Recent binding studies of polypeptide hormones (for review, see ref. 28) have emphasized the difficulty of assimilating binding sites to the functional receptors involved in adenylate cyclase activation as well as the importance of measuring both processes under strictly identical conditions. This is the first report for which labeled *beta* antagonist binding and measurement of adenylate cyclase activities were carried out using the same concentrations of ATP and ATP-regenerating system. It seems particularly important to include ATP in binding determinations, in view of the possible role of this nucleotide in both the coupling and desensitization processes (29, 30). Furthermore, adenylate cyclase activities are generally determined by the accumulation of cAMP throughout the entire incubation period, whereas binding is measured at the end of this period. Therefore, if agonist degradation, desensitization, or other processes occur, direct comparison between binding and adenylate cyclase activation is no longer possible. We avoided this obstacle by measuring binding at 10 min and adenylate cyclase activity from 9 to 11 min after the beginning of incubation. (−)-[^{3}H]Dihydroalprenolol interacts with only one category of binding sites (K_D* = 5.7 ± 0.9 nM; N = 5), as indicated by the presence of a single slope both in the Scatchard plot and in the semilogarithmic plot of the dissociation process. This affinity is very close to that reported for the (−)-[^{3}H]dihydroalprenolol binding sites in other systems (from 5 to 15 nM) (for review, see ref. 31). Using [^{125}I]hydroxybenzylpindolol, Maguire *et al.* (12) reported the presence in C6 GT 1A glioma cells of two categories of binding sites with the same affinity, but only one was related to the *beta* receptor. This difference might be due either to a difference between the C6 glioma cell clones used by the two laboratories or to the greater specificity of (−)-[^{3}H]dihydroalprenolol as a *beta* adrenergic ligand.

The binding capacity of the crude membrane fraction used in this study varied from 0.39 to 0.55 pmole/mg of protein, a value compatible with the capacity of various other cell membranes for *beta* receptors (for reviews, see refs. 17 and 31) or hormonal receptors (for review, see ref. 28). The C6 glioma cells used in this study contained about 10,000 (−)-[^{3}H]dihydroalprenolol binding sites, a figure which compares well with the 4000 [^{125}I]hydroxybenzylpindolol sites reported by Maguire *et al.* (12) in a C6 GT 1A clone. Furthermore, neuroblastoma cells which did not contain isoproterenol-sensitive adenylate cyclase did not specifically bind (−)-[^{3}H]dihydroalprenolol (Table 1).

We did not detect negative cooperativity between (−)-[^{3}H]dihydroalprenolol bind-

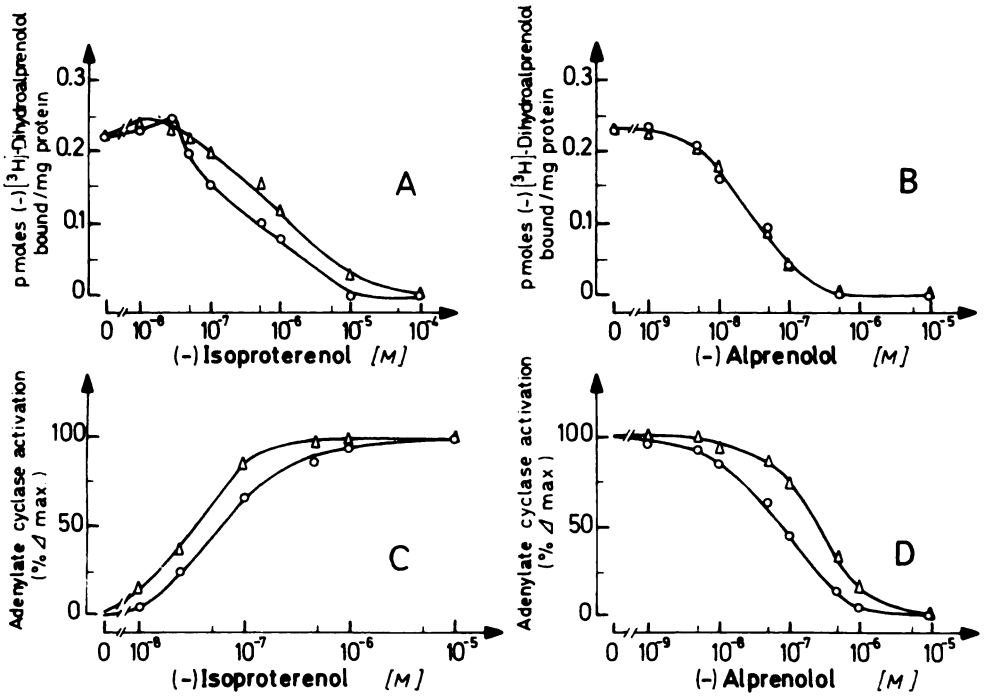

FIG. 9. *Effect of Gpp(NH)p on affinity constants for binding and adenylate cyclase of (−)-alprenolol and (−)-isoproterenol*

The membrane concentration was 35 μg of protein in 50 μl of solution. The (−)-[^{3}H]dihydroalprenolol concentration used for A and B was 9.3 nM. The (−)-isoproterenol concentration for D was 500 nM. The basal adenylate cyclase activities were 41 and 24 pmoles/2 min/mg of protein in the presence and absence of Gpp(NH)p (10 μM), respectively. The corresponding activities in the presence of (−)-isoproterenol (0.1 mM) were 115 and 268 pmoles/2 min/mg of protein. A. Effect of Gpp(NH)p on (−)-isoproterenol binding. The K_D values for (−)-isoproterenol were 380 and 120 nM in the presence and absence of Gpp(NH)p, respectively. B. Effect of Gpp(NH)p on (−)-alprenolol binding. The K_D value for unlabeled (−)-alprenolol was 10 nM in both the presence and absence of Gpp(NH)p. C. Effect of Gpp(NH)p on the $K_{A\ app}$ for (−)-isoproterenol activation of adenylate cyclase. The $K_{A\ app}$ values were 34 and 50 nM in the presence and absence of Gpp(NH)p, respectively. D. Effect of Gpp(NH)p on the $K_{I\ app}$ for (−)-alprenolol inhibition of (−)-isoproterenol-activated adenylate cyclase. Results obtained with or without Gpp(NH)p were indicated by (△) and (○) respectively, in A, B, C and D.

ing sites, an observation which differs from those reported for other systems (7, 32). However, the increase in (−)-[^{3}H]dihydroalprenolol binding observed when low concentrations of (−)-isoproterenol were added might indicate positive cooperativity between sites when both agonist and antagonist are present. This observation is under further investigation.

The order of potency of agonists as regards their ability both to compete with (−)-[^{3}H]dihydroalprenolol for binding sites and to stimulate adenylate cyclase was (−)-isoproterenol > norepinephrine ≅ epinephrine and is typical of *beta*$_1$ adrenergic specificity (33). Whatever the *beta* adrenergic agonist considered, the K_D binding/$K_{A\ app}$ ratio for adenylate cyclase activation was constant within the same experiment and equal to 3 (Figs. 4C, 5A, and 6, inset) or 2.4 (Fig. 9). As reported for the glucagon (15) and vasopressin (16) receptor-adenylate cyclase systems, this might indicate that the relationship between binding and adenylate cyclase activation is nonlinear (see also Fig. 10). From the experiments of Figs. 4B and 5A, it may be seen that, in the presence of 13.6 nM (−)-[^{3}H]dihydroalprenolol, total occupation of the binding sites by (−)-isoproterenol was

TABLE 3

Effects of Gpp(NH)p and ATP on affinity constants of (−)-alprenolol and (−)-isoproterenol for binding and adenylate cyclase

Assay conditions were the same as described in Fig. 9. ATP did not change (−)-[^{3}H]dihydroalprenolol binding capacity.

Expt.	Gpp(NH)p (10 μM)	ATP (200 μM)	K_D (−)-alprenolol	K_D (−)-isoproterenol	$K_{A\ app}$ (−)-isoproterenol
			nM	*nM*	*nM*
1	−	+	8.1	130	63
	+	+	8.1	560	33
2	−	+	10	120	50
	+	+	10	380	34
3	−	+	8.8	170	50
	+	+	8.8	300	30
	+	−	8.8	300	
	−	−	8.8	170	

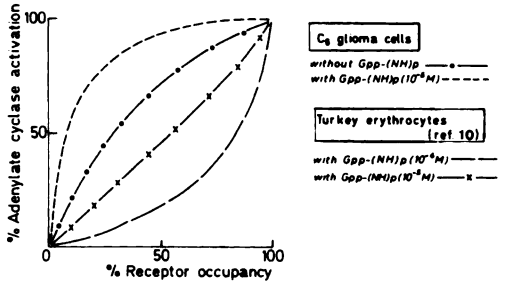

FIG. 10. *Effect of Gpp(NH)p on relationship between receptor occupancy and adenylate cyclase activation*

In C6 glioma cells the relationship were calculated on the basis of the affinity constants found in the experiment of Fig. 9, assuming that binding of (−)-isoproterenol and adenylate cyclase activation by (−)-isoproterenol followed Michaelis-Menten kinetics. In turkey erythrocytes the relationships are those found by Brown *et al.* (10).

obtained with a concentration of 10 μM, and 100% activation of the adenylate cyclase by (−)-isoproterenol was also reached at 10 μM. Occupation of the entire population of binding sites is therefore required to obtain 100% stimulation.

The order of potency of several *beta* antagonists as regards their ability to compete with (−)-[^{3}H]dihydroalprenolol binding sites and inhibit the (−)-isoproterenol-activated adenylate cyclase was hydroxybenzylpindolol > pindolol ≅ (−)-propranolol > (±)-propranolol ≅ (−)-alprenolol > dichloroisoproterenol > (+)-propranolol, which is exactly the order of potency found by Maguire *et al.* (12) using [^{125}I]hydroxybenzylpindolol as a tracer. The *alpha* adrenergic antagonists (phentolamine and phenoxybenzamine) and the dopamine receptor antagonists (fluphenazine and other neuroleptics) were unable to interact with either the binding sites or the adenylate cyclase system. This correlates well with the observation that neuroleptics were unable to inhibit the *beta*-sensitive adenylate cyclase in the central nervous system (34–36). The nonlinear relationship between binding site occupation and adenylate cyclase activation was confirmed by the value of 1.7 found for the ratio $K_D/K_{I\ app}$ (Figs. 4C, 5B, and 7, inset).

It was recently suggested (37) that purified ubiquitin [a single band was detected on polyacrylamide gel electrophoresis (38)], a naturally occurring polypeptide, might be a *beta* adrenergic agonist. We found ubiquitin to be totally ineffective both in interacting with binding sites and in affecting adenylate cyclase activity.

(−)-[^{3}H]Dihydroalprenolol binding was very fast (less than 1 min in the presence of 44 nM ligand) and totally reversible. The dissociation rate constant was equal to 0.35 min^{-1}. Since the dissociation constant for (−)-[^{3}H]dihydroalprenolol was 5.7 ± 0.9 nM (N = 5), it was possible to compute an association rate constant of 6×10^7 M^{-1} min^{-1} and therefore a $t_{1/2}$ of association of 14 sec.

When the (−)-[^{3}H]dihydroalprenolol binding sites were free, (−)-isoproterenol (0.1 mM) activated the adenylate cyclase instantaneously (Fig. 2A). On the other hand, in our experiment in which the binding sites were already occupied by the *beta* antagonist (−)-[^{3}H]dihydroalprenolol, adenylate cyclase activation by (−)-isoproterenol (0.1 mM) required 10 min and followed a kinetic pathway parallel to that of (−)-isoproterenol interaction with the (−)-[^{3}H]dihydroalprenolol binding sites (Fig. 2A and B). Consequently this interaction is the rate-limiting step in adenylate cyclase activation.

Thus equilibrium and rate studies show that (−)-[^{3}H]dihydroalprenolol binding

sites are the functional *beta*$_1$ receptor sites involved in adenylate cyclase stimulation.

As shown in Figs. 2A and 8B, there was a 25% time-dependent decrease in the steady-state rate of hormone-stimulated adenylate cyclase activity. This could be due either to hormone-dependent desensitization or to a burst in the hormone-activated state of the enzyme. Further experiments will obviously be necessary to understand this phenomenon.

As shown in Figs. 8 and 9 and Table 2, four different states of the adenylate cyclase system can be obtained: (a) a basal state (I), (b) an agonist-activated state (II), which is reversible and results from "coupling" of the agonist-receptor complex and the adenylate cylase, (c) a Gpp(NH)p-activated state formed in the absence of an agonist (III), and (d) a Gpp(NH)p-activated state formed in the presence of an agonist (IV). States III and IV are irreversible. The relative activities of all four states at equilibrium were I < III < IV < II. The agonist-activated state had a lower activity when formed in the presence of Gpp(NH)p. In all other hormonal systems so far described, the opposite observation was made (e.g., refs. 39, 40). However, GTP was shown to reduce the activity of vasopressin-activated adenylate cyclase in beef kidney plasma membrane (41).

Gpp(NH)p reduces the apparent affinity of the *beta* receptor for the agonist, (−)-isoproterenol, without affecting the apparent affinity for the *beta* antagonist, (−)-alprenolol (Fig. 9). This selective effect on agonist affinity makes direct action of Gpp(NH)p on receptor conformation unlikely. Furthermore, such direct action by Gpp(NH)p would not explain why its presence reduced the apparent activation constant of the adenylate cyclase system for (−)-isoproterenol. On the contrary, it is possible that Gpp(NH)p could indirectly modify the kinetics of agonist-receptor interaction by affecting one of the steps involved in the adenylate cyclase activation that follow such interaction. This assumption is more likely, since the effect of Gpp(NH)p on the apparent affinity of the *beta* receptor for its ligand is only observed in the case of "coupling" between the receptor and the adenylate cyclase. This effect was not apparent either when the ligand was an antagonist or when the *beta* receptor was solubilized (20). Whatever the mechanism involved, the presence of Gpp(NH)p increases the efficiency of coupling between hormone receptor occupancy and adenylate cyclase activation (Fig. 10).

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