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Efficiency of Coupling between the Beta Adrenergic Receptor and Adenylate Cyclase

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SUMMARY

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Under experimental conditions in which the coupling between the *beta* adrenergic receptor and adenylate cyclase was altered in S49 lymphoma cell membranes, changes were observed in the ratio of K_D to K_{act} for *beta* adrenergic agonists and in the effects of guanine nucleotide on the binding of agonists. The K_D for isoproterenol binding to the *beta* adrenergic receptor was greater than the K_{act} for enzyme activation, despite the use of a simultaneous assay technique. The ratio of K_D to K_{act} was decreased under conditions of less efficient coupling, such as in the presence of filipin or stimulation by partial agonists. However, cholera toxin treatment, which improves coupling efficiency, increased the ratio of K_D to K_{act} for both full and partial agonists. The ability of guanine nucleotides to decrease the affinity of the *beta* adrenergic receptor for agonists was lost when membranes were treated with filipin or 10 mM *N*-ethylmaleimide. However, 1 mM *N*-ethylmaleimide, a concentration sufficient to inhibit over 90% of adenylate cyclase activity in this membrane preparation, was ineffective in altering the binding characteristics. The effect of guanine nucleotide can thus be observed in the absence of a functional catalytic unit of adenylate cyclase.

INTRODUCTION

The binding of appropriate ligands to *beta* adrenergic receptors has been studied in a variety of normal and cultured cells. In a recent attempt to review this literature (1), we noted major differences between certain experimental systems. Among these are the presence or absence of an action of guanine nucleotides to decrease the affinity of agonists for the receptor and a marked variation in the ratio of agonist required to bind to the receptor (K_D) to that necessary for activation of adenylate cyclase (K_{act}). Maguire *et al.* defined a "well-coupled" receptor-adenylate cyclase system as one in which there was a high ratio of cyclic 3′,5′-AMP synthesized in response to agonist to the number of receptors occupied by saturating concentrations of the ligand (1). By inspection of data derived from a number of systems and under different conditions, it appeared that such "well-coupled" systems were those that also were characterized by a high K_D/K_{act} ratio and by the presence of an effect of guanine nucleotide on agonist binding. Since we prefer to believe that all receptor-adenylate cyclase systems are organized in a fundamentally similar manner, the present investigation

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was undertaken to ascertain whether experimental manipulation of coupling between receptor and adenylate cyclase could result in alterations in K_D/K_{act} and in the effect of guanine nucleotides.

METHODS AND MATERIALS

Cell culture and membrane preparation. S49 lymphoma cells were grown to a density of approximately 3×10^6/ml in 8-liter spinner flasks in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum. Cells were harvested and disrupted by nitrogen cavitation, and a relatively purified fraction of plasma membranes was prepared as described previously (2). All experiments reported here were performed with this membrane fraction.

Adenylate cyclase and beta adrenergic receptor binding activities. When adenylate cyclase was assayed alone, the method was the same as described previously (2), and the product was isolated by the method of Salomon *et al.* (3). Binding of ligands to the *beta* adrenergic receptor was quantified by assessment of their ability to compete for binding sites with [^{125}I]iodohydroxybenzylpindolol, also as described previously (2, 4). When detailed comparisons of agonist binding and enzyme activation were performed, the procedures were modified so that the assays were performed concurrently on the same samples. In this case assays were initiated by the addition of membranes (10–15 μg of protein) to a solution (140 μl) of sodium HEPES,[3] pH 8, 50 mM; ATP, 0.5 mM; $MgCl_2$, 10 mM; potassium phosphoenolpyruvate, 3 mM; pyruvate kinase, 0.1 mg/ml; Ro 20-1724, 0.1 mM; EDTA, 1 mM; bovine serum albumin, 1 mg/ml; ascorbic acid, 0.1 mM; and [^{125}I]iodohydroxybenzylpindolol (100–120 pM). Except where indicated, GTP was included at 0.1 mM and adrenergic agonists or antagonists were present as described. For the determination of nonspecific binding, propranolol was included at 1 μM. Specific binding is defined as the difference between total and nonspecific binding and represents approximately 95% of the total radioactive ligand bound under these conditions. Binding equilibrium was achieved by 40 min at 30°; at this time 0.75 μCi of [α-^{32}P]-ATP (in 10 μl) was added for an additional 20 min of incubation. The adenylate cyclase assay was then terminated by removal of a 75-μl aliquot, which was processed in the usual manner (2). The remainder of the reaction mixture was diluted and filtered by the technique previously described (4) to assess the binding of [^{125}I]iodohydroxybenzylpindolol. The usual maximal level of isoproterenol-stimulated adenylate cyclase activity in this assay was 250 pmoles/min/mg, and an average preparation contained 200 fmoles/mg of [^{125}I]iodohydroxybenzylpindolol binding sites.

Materials. Most reagents were obtained from sources listed previously (2). (−)-Isoproterenol was purchased from Sigma. Other adrenergic agents were obtained from the following sources: (±)-soterenol, from Mead Johnson; dobutamine, from Eli Lilly; (±)-ephedrine, from Merck; (±)-terbutaline, from Astra Pharmaceuticals; (±)-metaproterenol, from Boehringer/Ingelheim; and (−)-salbutamol, from Allen and Hansbury Research. *N*-Ethylmaleimide was purchased from Sigma, and dithiothreitol was obtained from Calbiochem. Ro 20-1724 was donated by H. Sheppard of Hoffmann–La Roche, and filipin (66% pure) was obtained from G. B. Whitfield, Jr., of the Upjohn Company.

RESULTS

Receptor-effector coupling with isoproterenol. The discrepant relationship between the K_D and the K_{act} for isoproterenol in S49 cell plasma membrane preparations (2) was verified using the simultaneous binding and adenylate cyclase assay technique. The specific binding of [^{125}I]iodohydroxybenzylpindolol to S49 cell plasma membranes appeared to reach equilibrium by approximately 40 min of incubation at 30° in the presence and absence of 10 μM isoproterenol (Fig. 1A). Because the agonist concentration was five orders of magnitude greater than that of the radioactive antagonist ligand, the former was presumed to

[3] The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazine-2-ethanesulfonate; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; UNC, the uncoupled phenotype in S49 lymphoma cells; AC$^-$, the adenylate cyclase activity-deficient phenotype in S49 lymphoma cells.

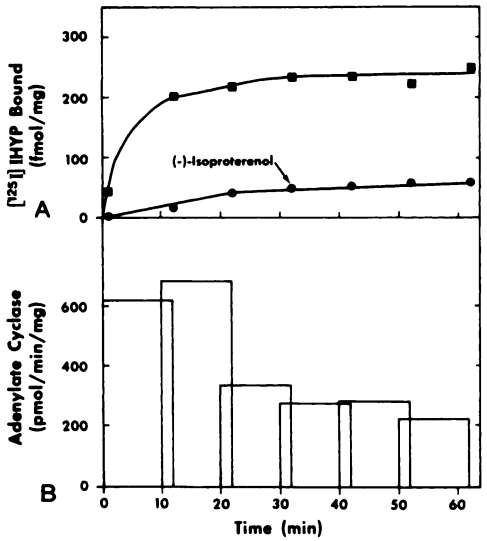

FIG. 1. *Time course for binding of [^{125}I]iodohydroxybenzylpindolol ([^{125}I]IHYP) and concurrent stimulation of adenylate cyclase*

At zero time, purified membranes were added to the reaction mixture in the presence or absence of 10 μM (−)-isoproterenol. At the times indicated, 140-μl aliquots were transferred to tubes containing 10 μl of [^{32}P]ATP for an additional 12-min incubation. The reaction was terminated for binding and enzyme determinations as described in the text. A. Specific [^{125}I]IHYP bound in the absence and presence of 10 μM (−)-isoproterenol. B. Isoproterenol-stimulated adenylate cyclase activity for overlapping 12-min intervals.

be in equilibrium essentially instantaneously; competition between the two ligands then resulted in the displacement of the agonist by the labeled antagonist to an extent dependent on their relative affinities and concentrations. During the interval when the binding of [^{125}I]iodohydroxybenzylpindolol was at equilibrium (40–60 min), the rate of isoproterenol-stimulated adenylate cyclase activity was essentially constant (Fig. 1B). This rate was, however, somewhat less than that observed during the first 20 min of incubation. Only a portion of this reduction can be ascribed to the binding of the antagonist ligand. Both catecholamine- and NaF-stimulated enzyme activities were decreased approximately 25% following incubation at 30° for 40 min (not shown). However the concentration dependence for activation of the enzyme by isoproterenol was the same when assayed from 0 to 20 min or from 40 to 60 min.

In keeping with our earlier results (2), the isotherms for binding and for activation of adenylate cyclase by (−)-isoproterenol do not correspond, despite the simultaneous assay technique (Fig. 2A). In six exper-

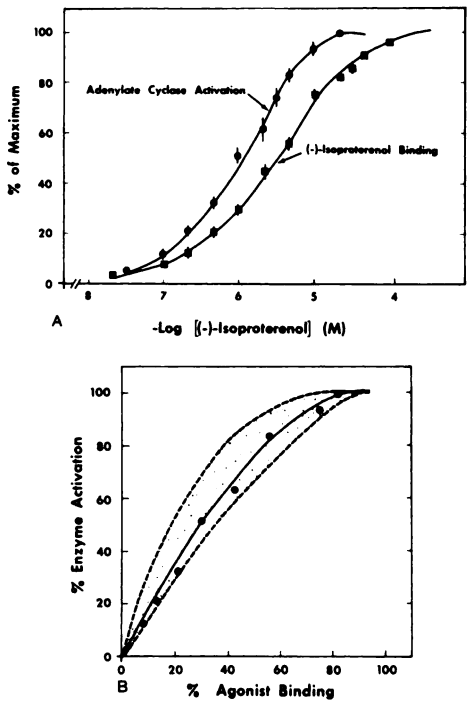

FIG. 2. *Relationship between (−)-isoproterenol binding to beta adrenergic receptor and adenylate cyclase activation*

A. Binding and enzyme activation isotherms for (−)-isoproterenol. The points represent means ± standard errors for six experiments. The simultaneous assay technique was used. Adenylate cyclase activation is the activity measured in the presence of agonist plus GTP, minus the basal level (GTP alone). The average maximal enzyme activity was 250 pmoles/min/mg. The average number of [^{125}I]iodohydroxybenzylpindolol binding sites was 200 fmoles/mg. Values of K_D and K_{act} for isoproterenol reported in the text were calculated from these data by correction for the competitive effect of [^{125}I]iodohydroxybenzylpindolol. K_D or K_{act} (K) is related to the concentration of isoproterenol required to inhibit binding by 50% or to stimulate adenylate cyclase half-maximally ($[L]_{1/2}$) by the equation $K = [L]_{1/2}(1 - f)$, where f is the fractional degree of saturation of receptor sites by [^{125}I]iodohydroxybenzylpindolol in the absence of competing ligand. A value of 90 pM was used for the K_D of the labeled ligand.

B. The data have been replotted from Fig. 2A. The stippled portion represents the range of points taken from the six experiments.

iments, the average K_D for isoproterenol in the presence of GTP was 1.3 ± 0.1 μM, with a Hill coefficient of 0.82, while the value of K_{act} was 0.43 ± 0.07 μM, with a Hill coefficient of 0.95. The K_D/K_{act} ratio was thus 3; in individual experiments it ranged from 2.2 to values greater than 5.

When the data of Fig. 2A are replotted as a graph of percentage of maximal effect against percentage of maximal binding, the resultant line lies above the diagonal (slope = 1) and is not linear (Fig. 2B). The initial slope is near 2; in this membrane preparation this corresponds to an approximate value of 3×10^3 molecules of cyclic AMP synthesized per minute in response to isoproterenol for each molecule of agonist bound. As the concentration of agonist is increased, this value falls by a factor of at least 2. Experimentally it is very difficult to determine whether further stimulation of adenylate cyclase occurs as agonist binds to the last fraction of receptors.

Manipulation of coupling efficiency. The polyene antibiotic filipin has been shown to cause a relatively selective loss of hormonal sensitivity of adenylate cyclase (5–7). The data of Fig. 3 verify this effect for S49 cell plasma membranes. These concentrations of filipin did not alter the binding of [^{125}I]-iodohydroxybenzylpindolol. In contrast to previous reports, filipin was consistently observed to have a stimulatory effect on NaF-stimulated enzyme activity at low concentrations.

At concentrations of filipin that only partially inhibited isoproterenol-stimulated adenylate cyclase, the stoichiometry between receptor occupancy by agonist and enzyme activation was altered (Fig. 4). There was strict correspondence between agonist binding and effect; the value of K_{act} became larger, and both K_D and K_{act} were approximately 1 μM (see Fig. 5). In one of the three experiments in Fig. 4, the maximal response to isoproterenol was not reduced, although the value of K_{act} was increased. As the system becomes uncoupled, the sequence of events thus appears to involve first a lowered value of K_D/K_{act}, followed by a diminished capacity to respond to an agonist.

Another property hypothesized to be typical of well-coupled systems is the ability of guanine nucleotides to cause an observable decrease in the affinity of agonists for the receptor. This effect has been observed in several systems where K_D/K_{act} is usually 1 or greater (2, 8, 9), and it is not seen in several others where this ratio is less than 1 (1, 10). Furthermore, there is no observable effect of guanine nucleotides on ago-

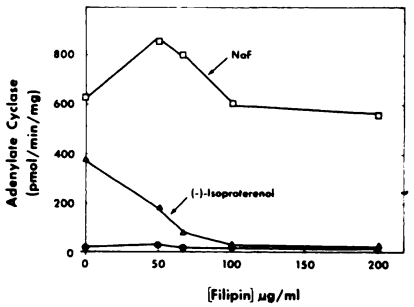

FIG. 3. *Activity of adenylate cyclase in the presence of filipin*

Adenylate cyclase activity was measured as described in the text for simultaneous enzyme and binding measurements. The concentration of (−)-isoproterenol was 50 μM, and that of NaF was 10 mM. Filipin was included in the assay mixture, and reactions were started by the addition of membranes.

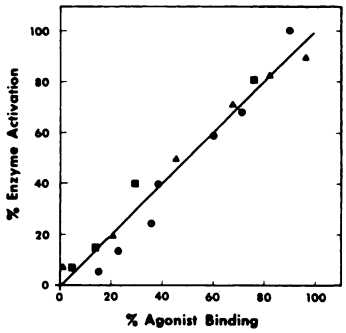

FIG. 4. *Effect of filipin on relationship between (−)-isoproterenol binding to beta adrenergic receptor and adenylate cyclase activation*

Adenylate cyclase activity was assayed as described in the text for simultaneous enzyme and binding measurements. Filipin was present in the reaction mixture at a final concentration of 36 or 40 μg/ml—chosen to attempt to uncouple the system partially. The data points were derived from three separate experiments, wherein the maximal effect of isoproterenol (compared with untreated samples) was 44% (△), 90% (●), and 100% (■) of untreated controls. The slope of the regression line through these points is 1.03 ($r = 0.97$).

nist binding in two types of S49 cell variants: an uncoupled clone, which has both enzyme and receptor but in which there is no stimulation of enzyme activity by adrenergic agonists (11), and a clone that lacks detectable adenylate cyclase activity (2). In both these variants the binding isotherm in the absence of guanine nucleotide is shifted to the right and assumes the position and shape normally observed only in the presence of nucleotide; addition of nucleotide has no further effect. A paradox thus seems to prevail: uncoupled systems show agonist binding characteristics in the absence of nucleotide that resemble those normally seen only when the regulatory nucleotide necessary for coupling is present.

With the hope of shedding some light on this apparent paradox, we examined the effects of filipin and *N*-ethylmaleimide on the binding of agonists to the *beta* adrenergic receptor, since membranes exposed to these compounds resemble those derived from UNC and AC^- variants, respectively. The data of Fig. 5 indicate that the guanine nucleotide effect on binding is lost following exposure of membranes to concentrations of filipin that partially inhibit the responsiveness of adenylate cyclase to catecholamines. Thus the negative heterotropic effect of guanine nucleotides is lost, the Hill coefficient for the binding of isoproterenol is elevated to a value near 1, and K_D is increased to a value typical of that usually seen only in the presence of nucleotide.

Treatment of membranes with 10 mM *N*-

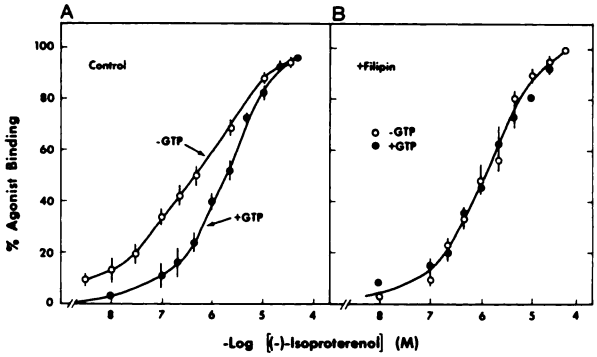

FIG. 5. *Effect of guanine nucleotides on (−)-isoproterenol binding to beta adrenergic receptor*
Data are means ± standard errors for three experiments. GTP was included as indicated at 50 μM. A. Control membranes. B. Filipin (50 μg/ml) was included in the reaction mixture.

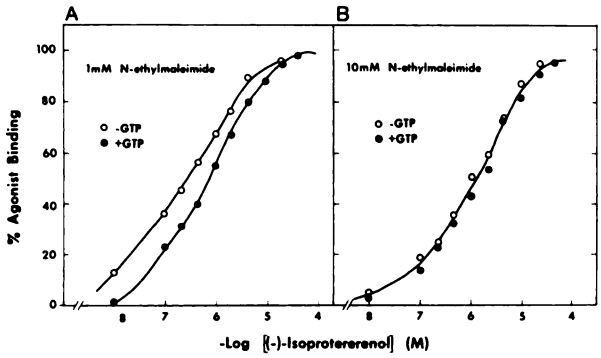

FIG. 6. *Effect of N-ethylmaleimide on (−)-isoproterenol binding to beta adrenergic receptor*
Membranes were incubated at 1 mg/ml in a buffer containing 20 mM HEPES (pH 8.0), 1 mM EDTA, 2 mM $MgCl_2$, and either 1 or 10 mM *N*-ethylmaleimide. After 20 min at 4°, the reaction was stopped by the addition of an equal concentration of dithiothreitol. After 10 min, the membrane suspension was diluted 5-fold with the above buffer, pelleted at 100,000 × *g* for 20 min, and resuspended to a final concentration of 1 mg/ml for assay. Either concentration of *N*-ethylmaleimide inhibited adenylate cyclase activity by more than 90% (not shown).

ethylmaleimide had a similar effect on binding (Fig. 6B). Membranes treated with this high concentration of the sulfhydryl reagent were indistinguishable, by the tests available, from membranes of adenylate cyclase-deficient S49 cells: they lacked detectable adenylate cyclase activity, the affinity of agonists for the *beta* adrenergic receptor was decreased (compared with wild-type membranes in the absence of GTP), and guanine nucleotides had no effect on the affinity of agonists. However, 1 mM *N*-ethylmaleimide failed to cause such an alteration in binding, even though this concentration was sufficient to inhibit more than 90% of the adenylate cyclase activity (Fig. 6A). The affinity of isoproterenol remained at the value characteristic of wild-type membranes, and the K_D for the agonist could still be raised by guanine nucleotides. Loss of adenylate cyclase activity itself is thus dissociated from loss of ability of guanine nucleotide to alter agonist affinity.

In contrast to the uncoupling effect of filipin, treatment of cells with cholera toxin prior to cell fractionation resulted in a membrane preparation with apparently improved coupling between receptor and enzyme. More than 10^4 molecules of cyclic AMP could be synthesized in response to the binding of a molecule of agonist ligand. The ratio of K_D to K_{act} was increased for two *beta* adrenergic agonists: from 3 to 8 for isoproterenol and from 2 to 20 for terbutaline (binding and enzyme activation isotherms not presented). This resulted from a decrease in K_{act}; the ability of the agonists to compete for [^{125}I]iodohydroxybenzylpindolol binding sites was unaltered. Furthermore, compounds that normally are only partial agonists were as efficacious as isoproterenol when tested with membranes from cholera toxin-treated cells (Table 1). It must be noted, however, that the maximal hormone-stimulated rate of cyclic AMP synthesis was now reduced because of the effect of the toxin on basal activity; this confuses the interpretation.

Finally, partial agonists were examined as another means of manipulating the efficiency of coupling between receptor and enzyme. We hypothesized that K_D/K_{act} would be lower for such agents. We also assumed that guanine nucleotides would have a diminished ability to raise the value of K_D for a partial agonist, since this effect of regulatory nucleotides is specific for agonists (8, 9). This latter phenomenon has already been demonstrated in the frog erythrocyte system by Lefkowitz *et al.* (9). While our expectations with regard to K_D/K_{act} were met to at least a certain extent (Table 2), it was not possible to demonstrate a strict correlation between K_D/K_{act} and efficacy, perhaps because of the errors inherent in the evaluation of the ratio. Furthermore, the range of values over which K_D/K_{act} has been observed to vary appears to be limited in S49 cell membranes, in that values substantially less than 1 have not been obtained.

TABLE 1

Effect of cholera toxin on ability of beta adrenergic agonists to stimulate adenylate cyclase

Wild-type S49 cells were harvested, washed, and suspended in growth medium without serum. The cells, at a density of 10^7/ml, were incubated for 2 hr at 37° with or without 1 μg/ml of cholera toxin. Membranes were then prepared as described (2), and assays for adenylate cyclase activity were performed. The concentration of agonist shown was that necessary to produce a maximal effect.

Agonist	Untreated			Cholera toxin-treated		
	Agonist concentration	Enzyme activity	Isoproterenol response	Agonist concentration	Enzyme activity	Isoproterenol response
	μM	*pmoles/min/mg*	%	*μM*	*pmoles/min/mg*	%
None		27	0		257	0
Isoproterenol	10	316	100	0.3	353	100
Salbutamol	30	224	68	10.0	366	113
Metaproterenol	300	214	65	100.0	379	127
Dobutamine	100	136	38	10.0	350	97

TABLE 2

Coupling efficiency with partial agonists

The compounds listed were tested in the simultaneous assay for adenylate cyclase activity and for the ability to bind to *beta* adrenergic receptors. Efficacy is defined as the relative ability to stimulate adenylate cyclase activity (isoproterenol = 1). The average maximal adenylate cyclase activity in the presence of isoproterenol was 260 pmoles/min/mg.

Compound	Efficacy	K_D/K_{act}
(−)-Isoproterenol	1.0	3.6
(±)-Terbutaline	0.75	1.8
(−)-Salbutamol	0.73	1.2
(±)-Soterenol	0.67	0.74
(±)-Metaproterenol	0.62	2.5
Dobutamine	0.26	2.6
Ephedrine	0.16	0.82

DISCUSSION

Among a variety of membrane preparations, the relationship between the K_D for binding of *beta* adrenergic agonists and the K_{act} for stimulation of adenylate cyclase by such compounds has been observed to vary over a wide range. For example, the ratios of K_D to K_{act} determined with membranes from turkey erythrocytes (10), rat heart (12), and rat liver (13) are less than 0.2; by contrast, the frog erythrocyte (14) and the S49 lymphoma cell (2) exhibit values of K_D/K_{act} that are equal to or greater than unity. Maguire *et al.* (1) noted a correlation between the value of K_D/K_{act} and the amount of cyclic AMP synthesized in response to the binding of a molecule of agonist. Thus the ratio of K_D to K_{act} may be indicative of the efficiency of coupling between the binding of agonists and the resultant stimulation of adenylate cyclase activity. Furthermore, it was noted that guanine nucleotides decreased the affinity of agonists for the *beta* adrenergic receptor only in systems that were defined as well coupled. For *beta* adrenergic agonists such shifts in affinity have been detected with several types of cultured cells (2, 8) and with frog erythrocytes (9), but the effect was not observed with turkey erythrocytes (10) or rat heart (1). The experimental goal of the present study was to alter the coupling efficiency of the adenylate cyclase system of S49 lymphoma cells and to observe the effects of such manipulation on the ratio of K_D to K_{act} and on the ability of guanine nucleotide to decrease the affinity of agonists.

The starting point for these experiments was a reassessment of the binding-effect relationship for isoproterenol. Previous attempts to define this relationship precisely, including our own, have all suffered from real or potential pitfalls. Thus incubation conditions for assessment of binding and enzyme activity must be the same, enzyme activity should be monitored at the same time as binding, and equilibrium assumptions must be met. For the latter reason, guanylyl imidodiphosphate should be avoided (2). The experiments above verify the fact that K_D/K_{act} for isoproterenol is greater than 1 for S49 cell membranes and emphasize the nonlinear relationship between binding to the receptor and activation of the enzyme. When only 10% of receptor sites are occupied by agonist, 20% of the maximal hormone-stimulated rate of adenylate cyclase activity has been achieved. This represents a rate of approximately 3000 molecules of cyclic AMP synthesized per minute per molecule of receptor occupied. At higher concentrations of agonist, binding is accompanied by a progressively smaller increment in enzymatic activity. The functional stoichiometric relationship between receptor and enzyme thus appears to be a variable.

Exposure of membranes to appropriate concentrations of the polyene filipin results in the selective loss of agonist-stimulated adenylate cyclase activity (5–7). Under conditions such that responses to isoproterenol were partially lost, K_D/K_{act} was reduced to 1. The ratio was lowered because of an increased value of K_{act}. Filipin can also cause an increase in the value of K_D, although this effect is not observed in the presence of GTP. The decrease in agonist affinity caused by filipin is of the same magnitude as that caused by GTP, and the ability of GTP to cause this change is lost concurrently. These results thus support the notion that loss of efficient coupling is accompanied by a lowered value of K_D/K_{act} and a loss of the ability of GTP to alter agonist binding. Membranes treated with appropriate concentrations of filipin closely

resemble those derived from UNC variant cells.

Data obtained with cholera toxin are also consistent with the proposed relationship between K_D/K_{act} and efficiency of coupling, since both are apparently increased in membranes prepared from cells partially activated by the toxin. The ratio is altered by a decrease in the value of K_{act}, and there is little or no effect of toxin treatment on hormone binding (2).

Despite attempts to obtain greater degrees of uncoupling with filipin or to examine weak partial agonists, K_D/K_{act} values substantially less than 1 have not been obtained with S49 cell membranes; such values are routinely observed, however, in other systems. The reason for this is not known, but it is conceivable that it lies with the number and arrangement of components in the individual membrane fragment in the preparation. K_D/K_{act} values less than 1 might result when there are multiple receptors per catalytic unit and the efficiency of coupling is poor. There are 500–1000 receptors per S49 cell, and electron micrographic observations of the membrane fragments utilized indicate that more than 1000 vesicles are probably produced from each cell. If the distribution of receptors is random, as seems reasonable for a cultured cell grown in suspension, few membrane vesicles would contain more than one receptor.

Treatment of membranes with high concentrations of *N*-ethylmaleimide (approximately 10 mM) results in changes that resemble those seen in adenylate cyclase-deficient variants of the S49 cell. There is no detectable adenylate cyclase activity, the affinity for agonists is reduced, and the ability of GTP to alter agonist affinity is lost. While one might assume that the common denominator to explain similar alterations in agonist binding in uncoupled cells or an adenylate cyclase-deficient cell involves loss of direct interaction between the receptor and the catalytic moiety, recent data indicate a more complex situation. First, studies of the ability of soluble, adenylate cyclase-containing detergent extracts to reconstitute responsiveness to *beta* adrenergic agonists in membranes derived from AC^- variants indicate that AC^- and UNC variants are not complementary; they appear to share a functional defect (15). Other data indicate that multiple components are necessary for guanylyl imidodiphosphate-, NaF-, or hormone-stimulated adenylate cyclase activity (16). One component, tentatively designated as the catalytic subunit, is considerably more sensitive to *N*-ethylmaleimide than are other components, although the latter are essential for the observation of any enzymatic activity. According to this model, 1 mM *N*-ethylmaleimide will inactivate the catalytic subunit without interfering with the function of the regulatory (coupling) factors. In the experiments described above this lower concentration of *N*-ethylmaleimide was shown not to alter agonist binding or to interfere with the effect of GTP on binding, despite essentially complete inactivation of adenylate cyclase activity. The properties of membranes treated with 1 mM *N*-ethylmaleimide are thus novel. These data are consistent with the hypothesis that the effect of GTP on agonist binding is due to interaction between the receptor and a nucleotide binding site on an intermediate component of the system. An active catalytic subunit appears not to be required. This hypothesis can be studied further in reconstituted systems of increasing definition.

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