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The Mechanism of Partial Agonism in the *Beta*-Receptor Dependent Adenylate Cyclase of Turkey Erythrocytes

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SUMMARY

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The efficacy of nine different β-agonists was measured, using three techniques in parallel: (a) the maximal steady state level of adenylate cyclase activity and (b) the first order rate constant describing the process of adenylate cyclase activation to its permanently active state, in the presence of GppNHp, and (c) the first order rate constant describing the process of cyclase deactivation, from its cAMP producing state to its inactive state. The efficacy of the β-agonist as measured by the steady-state adenylate cyclase induced activity is directly proportional to the first order rate constant characterizing the activation of the enzyme by the agonist bound receptor. In contrast, the first order rate constant characterizing the deactivation of the adenylate cyclase from its cAMP producing state to its inactive state is independent of the agonist and of the absolute level of steady-state adenylate cyclase specific activity, which differs for each agonist. These results strongly suggest that the efficacy of a β-agonist is solely dependent on the rate of adenylate cyclase activation by the agonist bound receptor.

INTRODUCTION

Partial agonism is a well-characterized phenomenon in pharmacology (for review see ref. 1). *Partial agonists* are ligands that elicit a submaximal response when bound to receptors at maximal occupancy, as compared to *full agonists* that elicit the maximal response when bound to the same set of receptors at maximal occupancy. Some of the receptors to hormones and neurotransmitters are coupled to adenylate cyclase and the activation of the latter occurs as a consequence of receptor occupancy by the agonist. Because much knowledge has been gained in recent years on receptor-dependent adenylate cyclases, especially on the mode of coupling between these two functional units, we chose to explore the difference between full agonists and partial agonists in biochemical terms. The system of choice is the turkey erythrocyte β-receptor dependent adenylate cyclase, for which the mode of coupling between the receptor and the catalytic moiety has been explored in detail (2–4). In the turkey erythrocyte cyclase, it was established (2–5) that the mode of cyclase activation by the agonist is by the "collision coupling" mechanism and

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can be described by the following scheme (4):

$$H\cdot R + E\cdot GTP \underset{k_1}{\rightarrow} [H\cdot R\cdot E\cdot GTP \leftrightarrows H\cdot R\cdot E'\cdot GTP] \quad (1)$$

$$\rightarrow H\cdot R + E'\cdot GTP \underset{k_{off}}{\rightarrow} E + GDP + P_i$$

where H is the agonist; R, the receptor; E, the enzyme; E', the activated form of the enzyme; k_1 the bimolecular rate constant that describes the collision between the enzyme and the agonist bound receptor; and k_{off} denotes the rate of enzyme deactivation from its active form E' to its inactive form E, concomitantly with the hydrolysis of GTP to GDP and P_i. The process of enzyme activation is bimolecular and occurs only when an agonist bound receptor and an enzyme moiety possessing GTP at the regulatory site are present. One can demonstrate (3, 4) that at steady state and at saturating agonist concentration and GTP concentration, the level of active enzyme is given by equation 2:

$$[E'] = \frac{[E_T]}{1 + k_{off}/k_{on}} = \frac{[E_T]}{1 + k_{off}/k_1[R_T]} \quad (2)$$

where $[E_T]$ the total enzyme concentration, k_{on} the rate constant of enzyme activation, k_{off} the rate constant of enzyme deactivation and $[R_T]$ the total receptor concentration. According to the model described in Equation 1 and analyzed by Equation 2, it is apparent that the maximal level of adenylate cyclase activity depends on the values of k_1 and k_{off}. The efficiency of an agonist depends on k_1, which expresses the frequency and the efficiency of the receptor to enzyme encounter, and on k_{off}, which is the turnover number of the GTPase step. Either or both of these constants may be agonist dependent. In the turkey erythrocyte system, it is possible to measure independently both, as well as [E']. Therefore, for the turkey system one can compare the experimentally measured steady-state level of cyclase activity, [E'], with the value predicted by Equation 2 for different agonists. This comparison conducted for a series of agonists and partial agonists is the subject of this communication. This comparison also serves to explore the validity of the model summarized in Equations 1 and 2, and described in detail elsewhere (3, 4).

MATERIALS AND METHODS

All solutions were prepared in doubly distilled water. Turkey erythrocyte membranes were prepared and stored as described earlier (6). Adenylate cyclase activity was determined by the method of Salomon *et al.* (7) and protein content by the Lowry technique (8). All agonist solutions were made freshly before use.

Measurements of k_{on}. The rate of enzyme activation was determined essentially as described earlier (4, 9), according to the following procedure. A mixture of agonist, GppNHp, α-[^{32}P]ATP, $MgCl_2$, Tris-HCl pH 7.4, buffer, theophylline, creatine phosphokinase and creatine phosphate and a membrane suspension in TME[3] buffer were incubated separately at 37° for 5 min. The reaction was initiated by the addition of the membranes to the mixture of reagents. The final concentrations in the reaction mixture were: 1 mM α[^{32}P]ATP (5–20 cpm/pmole), pH 7.4, 4 mM $MgCl_2$, 50 mM TME, 0.4 mg/ml theophylline, 5 units/assay creatine phosphokinase, 1 mM creatine phosphate, 1.0 to 1.2 mg membranes/ml and a saturating concentration of agonist. At chosen times 150 μl samples were withdrawn into a stopping solution consisting of 100 μl of 2% sodium dodecyl sulfate. The amount of [^{32}P]cAMP formed was determined by the Salomon method (7). Each experiment included a control in which 0.1 mM of *l*-epinephrine (full agonist) and a control with no agonist added. Under these conditions, in the presence of a nonhydrolyzable analogue of GTP (GppNHp), the adenylate cyclase is activated to a permanently (2) active form (k_{off} = 0 in Eq. 1 and 2). The kinetics of this process is first order (4) and therefore the time course of cAMP accumulation is given by the integral (4, 9) of the equation describing enzyme activation. The kinetics of cAMP accumulation is therefore described by:

[3] The abbreviations used are: TME, 50 mM Tris-HCl, pH 7.4, containing 2 mM $MgCl_2$ and 1 mM EDTA.

$$[cAMP]_t = k_{cat}[E_T] + \frac{k_{cat}[E_T]}{k_{obs}} \exp - [(k_{obs}t) - 1] \quad (3)$$

where $[cAMP]_t$ is the amount of cAMP at time t, subsequent to the initiation of the reaction, k_{cat} the turnover number of cyclase, and $[E_T]$ the total enzyme concentration. Therefore $k_{cat}[E_T]$ is the maximal adenylate cyclase specific activity, and k_{obs} the pseudo first order rate constant of enzyme activation. At saturating agonist concentration (4):

$$k_{obs} = k_1[R_T] = k_{on} \quad (4)$$

At nonsaturating concentration of agonist this parameter is multiplied by the fraction of receptors occupied by the agonist (4). Since in this study we were interested in obtaining the maximal values of k_{on}, saturating concentrations of agonists were used throughout. A computer fit of the data of cAMP accumulation with time to equation 3 yields directly (4, 9) k_{on} and $k_{cat}[E_T]$.

Measurement of k_{off}. The determination of k_{off} was performed by either the method of Tolkovsky and Levitzki (4) or the method of Cassel *et al.* (10). The latter method was found to be more reproducible and convenient and thus the results reported in this communication were all obtained by the latter methodology. The reaction mixture consisted of 0.3 mM ATP, pH 7.4, 4 mM $MgCl_2$, 50 mM TME buffer, pH 7.4, 1 μM GTP, 0.4 mg/ml theophylline, 1 mM creatine phosphate, 7.5 units creatine phosphokinase per assay and 0.1 mM of the agonist. All concentrations indicated are final concentrations. The membranes and reagents were preincubated separately at 37° before the reaction was initiated by the addition of membranes to the reagents. After 10 min a solution of $\alpha[^{32}P]$ATP possessing a specific radioactivity of 100 to 200 cpm/pmole and of *dl*-propranolol to yield a final concentration of 0.5 mM was added. At different time intervals a sample of 100 μl was removed into a 2% sodium dodecyl sulfate stopping solution and the amount of $[^{32}P]$cAMP was measured by the Salomon technique (7). Every experiment included a control with no agonist added. Using the scheme of the cyclase activation-deactivation cycle one can (4) show that the rate of enzyme deactivation is given by:

$$[E']_t = [E_0']\{\exp(-k_{off}t)\} \quad (5)$$

where $[E']$ is the concentration of active enzyme present at time t subsequent to propranolol addition, $[E_0']$ is the initial concentration of active enzyme at the time of propranolol addition ($t = 0$). Therefore, the time course at cAMP production subsequent to the addition of propranolol is given by:

$$[cAMP]_t = \frac{[E_0']}{k_{off}}[1 - \exp(-k_{off}t)] \quad (6)$$

At a long time subsequent to propranolol addition the value of cAMP produced will extrapolate to:

$$[cAMP]_{t\to\infty} = \frac{[E_0']}{k_{off}} \quad (7)$$

In the absence of propranolol addition the rate of cAMP production is linear with time and is given by:

$$[cAMP]_t = [E_0']t \quad (8)$$

After a certain time, t', the amount of cAMP made by the system without propranolol will be identical to the total amount of cAMP produced by the system to which propranolol was added. At this particular time, therefore:

$$[E_0']t' = \frac{[E_0']}{k_{off}} \quad (9)$$

and simplified:

$$t' = \frac{1}{k_{off}} \quad (10)$$

Thus by extrapolation of the plateau of the plot describing cAMP production by the propranolol-treated system to the straight line describing the time course of cAMP accumulation by the nontreated system, one can read the value of t' directly off the graph and therefore directly measure k_{off}. When the membranes possess basal activity, the latter must be subtracted from both curves before extrapolation. This subtrac-

TABLE 1

Kinetic Parameters for the Turkey Erythrocyte β-Receptor Dependent Adenylate Cyclase

	Steady-state specific activity		Maximal specific activity, $k_{cat}[E_T]$		Rate constant of activation, k_{on}				Rate constant of torn-off reaction, k_{off} at 37°	
					at 37°		at 30°, in Cis-vaccenic acid fluidized membranes			
	Absolute value	% of maximum	Absolute value	% of[a] maximum	Absolute value	% of maximum	Absolute value	% of maximum	Measured	Calculated
	pmoles cAMP/min/mg		*pmoles cAMP/min/mg*		min^{-1}		min^{-1}		min^{-1}	
l-epinephrine	84.3 ± 2.0	100	1008.4 ± 30	100	1.31 ± 0.090	100	3.0 ± 0.20	100	13.3 ± 1.5	14.83
dl-isoproterenol	82.0 ± 2.0	100	989.5 ± 30	100	1.31 ± 0.090	100	3.0 ± 0.20	100	13.3 ± 1.5	14.83
dl-p-hydroxybenzylisoproterenol	82.0 ± 2.0	100	1044 ± 30	100	1.32 ± 0.090	100	3.0 ± 0.20	100	ND	15.65
β-deoxyisoproterenol	30.0 ± 0.9	36	833.0 ± 25	82	0.50 ± 0.075	38	1.0 ± 0.065	33	14.0 ± 3.5	13.38
l-soterenol	17.0 ± 0.4	21	750.5 ± 20	74	0.38 ± 0.28	28	0.83 ± 0.060	28	13.0 ± 7.0	16.40
dopamine	18.5 ± 0.4	22	650 ± 20	71	0.33 ± 0.022	25	0.66 ± 0.045	22	15.0 ± 8.0	11.26
l-phenylephrine	18.5 ± 0.4	22	792.0 ± 25	78	0.29 ± 0.021	22	0.53 ± 0.040	18	ND	11.26
dl-salbutamol	13.6 ± 0.3	16	750.0 ± 25	74	0.22 ± 0.015	17	0.45 ± 0.040	15	ND	11.91
dl-benzylisoproterenol (MJ-9184-1)	12.4 ± 0.25	15	717.2 ± 20	71	0.38 ± 0.008	28	0.71 ± 0.055	24	ND	21.60
dl-metanephrine	0.0 ± 0.02	0	ND	ND	0.008	2.7	ND	ND	ND	ND
l-sotalol	0.0 ± 0.2	0	ND	ND	0.0	0	ND	ND	ND	ND

[a] In experiments with membranes treated with cis-vaccenic acid a very similar result was obtained. Namely, most partial agonists induce only 70 to 75% of the maximal specific activity induced by full agonists in the presence of GppNHp. The specific activities induced by either *l*-isoproterenol, *l*-epinephrine, *dl*-hydroxybenzylisoproterenol and *l*-norepinephrine was found to be 1950 ± 70 pmoles cAMP/min/mg at 30° compared to 1400 ± 50 to 1500 ± 50 pmoles cAMP/min/mg induced by the partial agonists listed in the table.

All the experimentally determined values reported are the mean of at least 3 determinations plus the standard error of the mean.

The procedures to measure cyclase steady state specific activity, k_{on} and k_{off} are given in the MATERIALS AND METHODS. Cis-vaccenic membranes were prepared as described in the experimental section. The particular batch of unfluidized membranes used for these experiments possessed a k_{on} value of 0.61 min^{-1}. k_{off} was calculated according to equation 2. E' was taken as the steady-state specific activity (leftmost column) and E_T the maximal specific activity in the presence of saturating concentrations of the agonist and GppNHp. This value was found to be 1020 ± 25 pmoles cAMP/min/mg at 37° for the unfluidized membranes and 2010 ± 100 pmoles cAMP/min/mg at 30° for the particular batch of cis-vaccenic acid treated membranes. The value of k_{on} was the experimentally measured value. Thus for each ligand the validity of equation 2 could be examined since all the parameters were measured independently.

tion is essential, especially in cases where the agonists are weak and produce little effect on the activation of the system. This procedure yielded inaccurate results for agonists that induce less than 30% of the effect induced by the full agonists.

Cis-Vaccenic acid treatment of the membranes. The application of cis-vaccenic acid on turkey erythrocyte membranes was conducted by the method of Orly and Schramm (11), as previously described (9, 12) and was conducted as follows: To 1.0 ml suspension of washed membranes at a final concentration of 3.0 mg/ml, 10 μl of 100 mM cis-vaccenic in absolute ethanol were added and the mixture was incubated for 10 min at 0–4°. The final concentration of ethanol in the cyclase assay never exceeded 0.5%; this concentration was found to be without effect on the cyclase reaction. Fluidization of the membranes increased the efficiency of receptor to enzyme coupling and also caused an increased specific activity (9, 12). Therefore, some of the low measurable activities were increased by fluidization without increasing temperature, which results in a slow irreversible inactivation over the long incubation times required especially for very weak agonists.

RESULTS

Determination of k_{on}, k_{off} and steady state agonist dependent specific activity. The steady state adenylate cyclase activity in the presence of full agonists and partial agonists is summarized in Table 1. In the same table are summarized the values of the rate constants of cyclase activation by the same β-agonist in the presence of GppNHp(k_{on}) and the rate constants of the enzyme deactivation, from its active cAMP-producing state to its inactive state (k_{off}). Some typical experiments for the determination of k_{on} and k_{off} are depicted in Figures 1 and 2. It must be emphasized that the determination of k_{off} is relatively accurate only for full agonists and quite inaccurate for partial agonists. This is due to the fact that the difference between the basal activity and the agonist dependent activity narrows as the agonist becomes weaker. Therefore, the fall in the rate of [^{32}P]cAMP accumulation subsequent to propranolol addition becomes less significant the weaker the agonist such that the determination of t' (see EXPERIMENTAL PROCEDURES) becomes less accurate. Other details are given in there and in the figure legends.

Calculation of k_{off}. Using the experimentally determined values of [E'] and k_{on}, one can calculate the values of k_{off} using equation 2 for each agonist, and compare them with the experimentally determined values of k_{off}, when available. This comparison is also summarized in Table 1.

The dependence of the maximal specific activity on the nature of the β-agonist. From Table 1 it is apparent that partial agonists induce 70 to 75% of the maximal specific activity induced by full agonists, in the presence of GppNHp. This point was checked thoroughly and is represented in Figure 3. Partial agonists consistantly induce a lower specific activity than full agonists, when incubated in the presence of GppNHp. A summary of seven different experiments on seven different batches of membranes is shown in Figure 3.

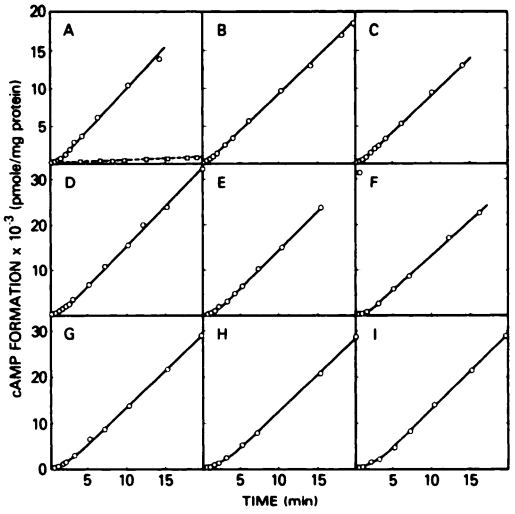

FIG. 1. *The activation of adenylate cyclase by full and partial agonists in the presence of GppNHp: determination of k_{on}*

Typical cAMP accumulation curves in the presence of different β-agonists and GppNHp at 37°. Experimental details are given in the text. A) *l*-epinephrine; B) *dl*-isoproterenol; C) *dl*-phydroxybenzyl-isoproterenol; D) β-deoxyisoproterenol; E) *l*-soterenol; F) *dl*-benzylsoterenol; G) dopamine; H) *l*-phenylephrine; I) *dl*-salbutamol. O——O = agonist plus GppNHp; □——□ GppNHp alone.

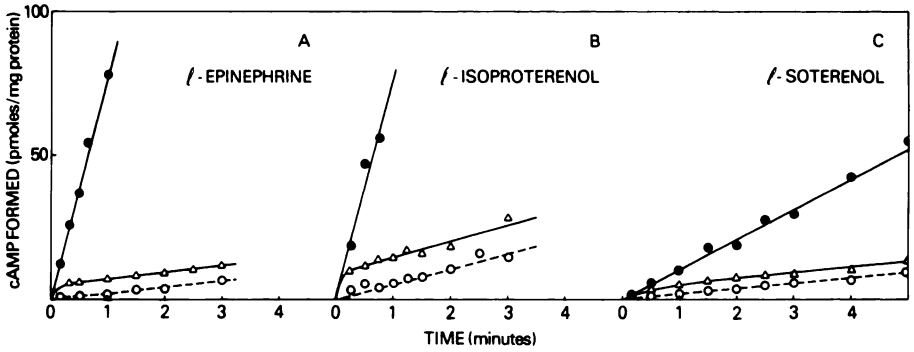

FIG. 2. *The rate of adenylate cyclase deactivation: the determination of k_{off}*

Typical experiments for the determination of k_{off} for full agonists and a partial agonist. Membranes at a final concentration of 6.25 mg/ml were incubated for 3 to 5 min at 37° with an activation mixture containing 0.3 mM ATP, 4 mM $MgCl_2$, 50 mM TME buffer 20 μM of the agonist, 1 μM GTP, 0.4 mg/ml theophyline, 1 mM creatine phosphate and 7.5 units/assay. At time zero ($t = 0$) ethanol free α-[^{32}P]ATP (100 to 200 cpm/pmole) were added with (△) or without (●) *dl*-propranolol (0.5 mM final concentration). A control experiment with no agonist was conducted in parallel (○). At different times a sample of 100 μl assay mixture was removed and added to a stopping solution. Further details are given in the MATERIALS AND METHODS.

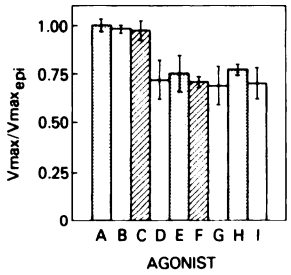

FIG. 3. *The maximal specific activities induced by β-agonists*

The results presented in this figure are from seven different experiments on a number of turkey erythrocyte membrane preparations. The bars indicate the standard error of the mean of triplicate determinations. A) *l*-epinephrine; B) *dl*-isoproterenol; C) *dl*-hydroxybenzylisoproterenol; D) β-deoxyisoproterenol; E) *l*-soterenol; F) *dl*-benzyl soterenol; G) dopamine; H) *l*-phenylephrine; I) *dl*-salbutamol.

The effect of partial agonists on full agonist plus GppNHp induced activity. Turkey erythrocyte adenylate cyclase was activated to its highest permanently active form by *l*-epinephrine and GppNHp and subsequently exposed to and assayed in the presence of the different partial agonists in the presence of GppNHp. The converse experiment was also performed: The cyclase was activated by partial β-agonists and GppNHp and subsequently incubated and assayed in the presence of a full agonist and saturating GppNHp. In the two types of experiments it was found that the specific activity of the system is determined by the ligand to which the system was exposed first.

For comparison, an experiment was performed in parallel with adenosine. It was found that *l*-epinephrine plus GppNHp elevates the maximal specific activity induced by adenosine and GppNHp to that typical of the *l*-epinephrine plus GppNHp induced activity confirming previous results (13).

DISCUSSION

The mechanism of partial agonism in the β-receptor dependent activation of adenylate cyclase. Equation 2 was shown to account rather well for the β-adrenergic receptor dependent cyclase activity and for the adenosine dependent cyclase activity in turkey erythrocyte membranes (4, 13). In this study we have attempted to determine whether certain ligands act as partial agonists because they induce a smaller k_{on} or a higher k_{off}. It can be seen that either mechanism can in principle account for different values of [E']: Since the ratio k_{on}/k_{off}, in fact, determines the fraction of the enzyme which is in the active state (Equation 2). It is evident from Table 1 and Figure 4 that the basic conclusion of this

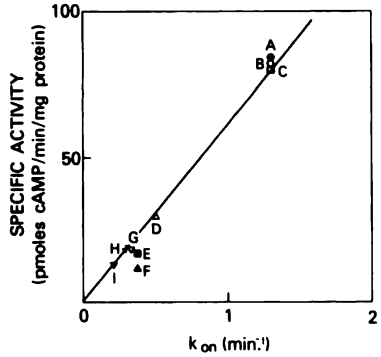

FIG. 4. *The correlation between steady-state adenylate cyclase specific activity and the rate constant of adenylate activation for different agonists*

All determinations were carried out at 37° as explained in the text. (A, ●) *l*-epinephrine; (B, ○) *dl*-isoproterenol; (C, □) *dl*-hydroxybenzylisoproterenol; (D, △) β-deoxyisoproterenol; (E, ■) *l*-soterenol; (F, ▲) *dl*-benzylsoterenol; (G, ▽) dopamine; (H, +) *l*-phenylephrine; (I, ▽) *dl*-salbutamol.

study is that the weaker the agonist, the lower is the value of k_{on} describing the rate of cyclase activation by the ligand bound receptor. The deactivation rate constant, on the other hand, k_{off} seems to be independent of the activating ligand. Since for each agonist it is clear (Table 1) that $k_{off} \gg k_{on}$, equation 2 can be approximated by:

$$[E'] = \frac{k_{on}}{k_{off}} [E_T] \qquad (11)$$

From the constancy of k_{off}, (Table 1), it is expected that the steady state level of adenylate cyclase activity induced by an agonist should be directly proportional on the value of k_{on}. Indeed, Figure 4, which summarizes the results for 9 different agonists, shows this relationship between [E'] and k_{on}. This linear dependence of (E') on k_{on} is preserved in cis-vaccenic acid treated membranes (data not shown).

The maximal specific activity induced by different agonists. The maximal specific activity k_{cat} [E_T] is obtained when all the enzyme pool is converted to the cAMP producing state (E'). This can be achieved upon incubating the enzyme system with the agonist in the presence of saturating concentration of a nonhydrolyzable GTP analogue such as GppNHp. We have found earlier (13) that adenosine plus GppNHp induce only 60 to 70% of the maximal specific activity induced by *l*-epinephrine and GppNHp. In the latter case we have shown that the addition of a full β-agonist plus GppNHp subsequent to the maximal activation by adenosine and GppNHp results in an additional activation of the "missing" 30 to 40% (13). The conclusion from this experiment (as well as from others) was that only 60 to 70% of the enzyme pool can be activated by the adenosine receptor, whereas 100% of the pool can be activated by the β-receptors and GppNHp. Surprisingly, we found in this study that a whole family of partial β-agonists can only induce 70 to 75% of the maximal specific activity induced by full agonists (Table 1, Fig. 3). However, unlike the adenosine case, the addition of a full β-agonist and GppNHp, subsequent to the maximal activation of the cyclase system by the partial agonist and GppNHp, does not result in the additional 25 to 30% increase in specific activity. Furthermore, the addition of a partial agonist plus GppNHp subsequent to the full activation by the full agonist plus GppNHp *does not* induce a decrease in the specific activity of the system. These two sets of observations indicate that (a) all of the cyclase pool is activated by either full or partial β-agonists and (b) the efficiency of the activated state induced by the partial agonists is 25 to 30% lower than that obtained by full agonists and GppNHp. It is not clear however, at this point, why, once the cyclase system is activated to its permanently active state by a partial agonist and GppNHp, it is "locked" into this state. Even the removal of the partial agonist and the exposure of this state to a full agonist and GppNHp does not induce the expected 25 to 30% increase in catalytic efficiency.

The collision coupling of cyclase to β-receptors. The collision coupling mechanism responsible for adenylate cyclase activation by the β-receptors in turkey erythrocytes depicted in Equation 1 is valid for full β-agonists as well as for partial agonists (4). As was shown here, the efficiency of the agonist is determined by k_{on}. The parameter k_{on} in itself is determined by two parameters: the relative receptor to enzyme

diffusion coefficient and the efficacy of the encounter between the two macromolecules. It is reasonable to assume that the diffusion coefficient of the receptor is independent of the ligand bound to it and that the efficacy of the encounter is ligand dependent. One can assume that the better the agonist the higher the fraction of the receptor molecules which are in the "active" conformation capable of activating the cyclase moiety once the two entities collide within the membrane matrix.

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