0026-895X/89/030437-09$02.00/0

MOLECULAR PHARMACOLOGY, 36:437-445

Evidence for the Role of Epinephrine Binding Frequency in Activation of Adenylate Cyclase

DOUGLAS STICKLE and ROGER BARBER

Laboratories of Cyclic Nucleotide Research, Graduate School of Biomedical Sciences, University of Texas Health Science Center, Houston, Texas 77225

Received May 2, 1989; Accepted June 23, 1989

SUMMARY

The binding of epinephrine to β-adrenergic receptors is a rapid-on, rapid-off process, such that at any level of receptor occupancy (defined as the fraction of time a receptor is bound or, alternatively, the probability that any particular receptor is bound at any given instant) the entire population of available receptors has periods of occupancy that occur at high frequency. While in the bound state, the receptor acts as a mobile catalyst for the activation of adenylate cyclase. Two processes, then, could conceivably contribute to the access of epinephrine-bound receptors to cyclase and the extent of cyclase activation for a given concentration of epinephrine: 1) the rapid switching of epinephrine among receptors ensures that discontinuous distributed regions of the cell surface experience agonist activity and 2) the mobility of the receptors (and GTP-binding protein) in the cell membrane makes it possible for one receptor to activate numerous GTP-binding protein-adenylate cyclase complexes. In principle, either effect can lead to a wide separation between the binding and response curves ($EC_{50} \ll K_d$). It has so far been assumed that mobility is able to account completely for the separation. The extent of the contribution of the process of agonist binding and unbinding to adenylate cyclase activation has not been demonstrated or quantified. Here we examine the distinction between binding frequency and receptor mobility contributions to adenylate cyclase activation in epinephrine-stimulated S49 lymphoma cells for which there is a 200-fold separation between the EC_{50} and K_d at 37° (EC_{50} = 10 nM, K_d = 2 μM). Experiments were designed to measure adenylate cyclase activation rates for a constant concentration of epinephrine-bound receptors but with variation of the absolute number of receptors involved in the activation. This was accomplished by blocking a portion of the receptor population with an antagonist (propranolol) that has a long occupancy half-life, while increasing the occupancy of the remaining receptors by compensating increases in epinephrine. With this protocol, a condition is approached in which receptor mobility alone is responsible for activation. This resulted in a 50% decrease in adenylate cyclase activity, compared with a control of 30 nM epinephrine. Thus, for epinephrine concentrations near the EC_{50}, the switching of epinephrine among the receptor population is necessary for >50% of the observed activity; it can be shown in conjunction that receptor mobility nonetheless accounts for the majority of the separation between the EC_{50} and the K_d. A theoretical analysis of the probability of cyclase activation based on the diffusion of receptors in the membrane gives a conclusion consistent with those derived from the experimental data.

The stimulation by epinephrine of adenylate cyclase in the S49 cell exhibits a wide separation between the EC_{50} (10 nM, the concentration at which activation is half-maximal) and the dissociation constant K_d (2 μM, the concentration at which receptors are occupied 50% of the time) (1). Thus, in this system receptors need to be occupied by epinephrine only a small fraction of the time in order to promote near-maximal activation. The separation between binding and response can be characterized by the pharmacological shift ratio (K_d/EC_{50}), which is related to the intrinsic G protein/adenylate cyclase-activating capability for β-adrenergic receptor agonists (2).

A separation between binding and activity can be explained by the collision coupling model of Tolkovsky and Levitzki (3). The model attributes the rate of cyclase activation to the frequency and efficiency of collisions between agonist-bound receptors and G protein, by which any one receptor can activate a number of G protein molecules due to the free mobility of each within the membrane. The rate of adenylate cyclase activation [kinetically equivalent to the rate of activation of G protein (4, 5)] is, therefore, according to this model, directly proportional to the number of agonist-bound receptors.

The collision coupling model does not make the distinction, however, between widely different circumstances in which the concentration of agonist-bound receptors is identical. Specifically, the model makes no distinction between the case of having some number (n) of receptors bound 100% of the time and the case of having a steady state number of receptors bound also equal to n, from among a larger population of N receptors,

This work was supported in part by National Institutes of Health Grant R01 NS21338.

ABBREVIATION: G protein, GTP-binding protein.

each bound only a fraction of the time (on average, n/N of the time). This is the difference between having high occupancy (the fraction of time a given receptor is bound) of a low absolute *number* of receptors and having a low receptor *occupancy*. A fractional occupancy, θ, of N receptors means not only that an average of $N\theta$ receptors are occupied at any one time, but also that, over a sufficiently long time period, all receptors are occupied for a fraction θ of the time. If the half-life of the receptor-agonist complex is short compared with an arbitrary time interval, then a fraction greater than θ of the receptors will carry an agonist for part of that time interval. Because at binding equilibrium the overall fractional occupancy by agonist is constant, rapid dissociation of agonist is equivalent to rapid switching of occupancy from one receptor to another. In this paper we will refer to this phenomenon as "switching."

In the case of n receptors bound 100% of the time, the receptor mobility, and therefore the receptor-enzyme collision rate, will be the rate-limiting step in the activation of G protein/adenylate cyclase. In comparison, activation involving N receptors may potentially be greater, depending on the frequency with which an individual receptor switches between the states of being bound and unbound, because a high binding frequency would ensure that agonist-bound receptor activity would be distributed among discontinuous separated regions of the cell surface (5). From the standpoint of an individual receptor, when the agonist binding frequency is high it is potentially less probable that it will "waste" collisions with G protein/adenylate cyclase molecules that it has already activated. The extent of the difference depends on the relative contributions of agonist binding frequency and receptor mobility to the activation.

In principle, then, part of the pharmacological shift ratio of a β-adrenergic agonist may be attributable to its binding frequency. Epinephrine has a relatively low affinity for β-adrenergic receptors and a 200-fold separation between EC_{50} and K_d in the intact S49 cell (1). Its binding is of a high enough frequency so that the individual rate constants for binding and unbinding have not been measured directly. The relative extent of the contribution of agonist binding frequency to the process of adenylate cyclase activation has not been determined or demonstrated for any agonist. Here we examine in detail the distinction between mobility and binding frequency contributions to cyclase activation rates, from both theoretical and experimental standpoints, in intact epinephrine-stimulated S49 lymphoma cells and quantify the contributions of each to the process of activation. A series of experiments was designed to measure the activation rate of adenylate cyclase as a function of the number of receptors accessible to agonist over the time course (1 min) of the experiment, while maintaining the concentration of agonist-bound receptors as a constant. This was accomplished by using increasing concentrations of the β-adrenergic receptor antagonist propranolol, which, because of its relatively long occupancy half-life, can effectively reduce the number of receptors available to epinephrine. The decreased responsiveness under this circumstance demonstrates that the high binding frequency of epinephrine contributes in part to the pharmacological shift ratio. Thus, epinephrine binding frequency appears to enhance its potency beyond the limitations prescribed by receptor mobility. A theoretical analysis of adenylate cyclase activation for the mobility-limited case supports this conclusion.

Experimental Procedures

Materials. (−)-Epinephrine bitartrate and (±)-propranolol were purchased from Sigma; metoprolol (5 mg/5 ml of H_2O) was purchased from Geigy; [8-^{3}H]adenine (17 Ci/mmol), [8-^{14}C]ATP (500 mCi/mmol), and [8-^{14}C]cyclic-AMP were purchased from ICN.

Cell culture. S49 murine lymphoma cells were kindly provided by Dr. Henry Bourne, University of California at San Francisco. Cells were grown in 1-liter roller bottles at 37° with Dulbecco's modified Eagle's medium (GIBCO) that was supplemented with antibiotics (GIBCO) and 5% horse serum. Cell density was maintained between 1.5 and 2.0 × 10^6 cells/ml by daily addition of fresh medium.

cAMP accumulation experiments. Cells were washed in serum-free Dulbecco's modified Eagle's medium, resuspended at a density of 20 × 10^6 cells/ml, and then incubated for 60 min at 37° in medium containing 10 μCi [^{3}H]adenine/ml. Adenine remaining in the medium was removed by washing and the cells were incubated in fresh medium for 30 min at 37°. After hormone addition, aliquots were removed and cells were collected by centrifugation and discarding of aspirated medium. Cell response was stopped after 1 min by addition of 5% trichloroacetic acid along with [^{14}C]ATP and [^{14}C]cAMP used to monitor recovery. Extracts were cleared of precipitated protein by centrifugation and were fractionated on Dowex 50 and alumina columns as previously described (6). Accumulation of cAMP during 1 min was measured as percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP:

$$\% \text{ conversion} = [^3\text{H}]\text{cAMP}/[([^3\text{H}]\text{cAMP} + [^3\text{H}]\text{ATP})$$

Experiments limiting receptor participation by addition of antagonist. The control experiment was a 1-min assay of cAMP accumulation during stimulation by a concentration of agonist (A) that was chosen to give >50% activation (f), given its approximate EC_{50} (10 nM), using

$$f = [A]/([A] + EC_{50})$$

Occupancy (θ) of β-adrenergic receptors for control was calculated, given K_{d_A} (2 μM), by

$$\theta_A = [A]/([A] + K_{d_A})$$

In subsequent experiments, the access of agonist to receptors was altered by the addition of β-adrenergic antagonist; constant agonist-receptor concentration was maintained by increasing both agonist and antagonist concentrations. In the presence of antagonist B, steady state occupancy by agonist is given by

$$\theta_A = [A]/K_{d_A}\,/(1 + [A]/K_{d_A} + [B]/K_{d_B})$$

Thus, for a given $[A]$, the $[B]$ required to maintain a fixed θ is given by

$$[B] = K_{d_B}\{[A](1 - \theta)/(\theta K_{d_A}) - 1\}$$

This calculated amount of antagonist was added 6 min before the beginning of the assay. At the concentration of antagonist used in these experiments, 6 min was sufficient for binding equilibrium to be obtained. To begin the assay, the required concentration of agonist was added; the assay was terminated by acid addition after 1 min.

Calculation of activation rate from cAMP accumulation. Maximum activity, V_{max}, for cyclase was calculated from the control experiment

$$V_{max} = V(1 + EC_{50}/[A])$$

or measured directly in an experiment using a saturating concentration of epinephrine. For subsequent points, the fractional activity f was calculated by

$$f = V/V_{max}$$

Because activity represents a steady state between processes of activation and inactivation (7), then

$$f = k_a/(k_a + k_i)$$

where k_a is the activation rate constant and k_i is the inactivation rate constant. Given f, the ratio k_a/k_i may be calculated:

$$k_a/k_i = f/(1 - f)$$

Results

Receptor participation limited by a slowly dissociating antagonist, propranolol. The experiments were designed to examine the activation rate of adenylate cyclase as a function of the number of receptors accessible to epinephrine over the time course (1 min) of the experiment, while maintaining the concentration of epinephrine-bound receptors as a constant. The access of epinephrine to receptors was altered by the addition of the β-adrenergic antagonist propranolol; constant epinephrine-receptor complex concentration was maintained by compensating increases in epinephrine.

The concentrations of epinephrine were set over a range from 30 nM (control) to 10 μM and the corresponding propranolol concentrations required to maintain constant overall epinephrine-bound receptor concentration were calculated as described in Experimental Procedures. The K_d for epinephrine (2 μM) was determined for intact S49 cells (1) using the nonequilibrium technique of Toews *et al.* (8). The value, therefore, is that for naive cells and should be the most appropriate value for the binding constant under the conditions of this experiment. The dissociation constant for propranolol for these cells was determined to be $K_d \simeq 650$ pM, with a half-life of the propranolol-receptor complex of 150 sec (data not shown). Because of its slow dissociation from receptors, the presence of propranolol under the conditions of this experiment was designed to effectively reduce the number of receptors available to epinephrine during the 1-min experiment, because a receptor that is bound to propranolol at the time of addition of epinephrine is likely to remain bound to propranolol during the entire 1-min assay. The restriction of epinephrine occupancy by this means to a smaller number of receptors reduced the activity of adenylate cyclase, compared with control of 30 nM epinephrine (Fig. 1).

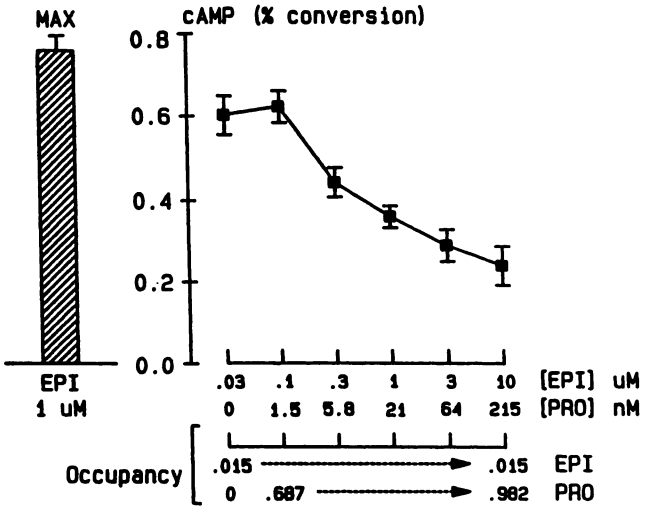

Fig. 1. cAMP accumulation (1-min percent conversion; average of triplicates ± standard error) stimulated by epinephrine (*EPI*) in the presence of varying concentrations of propranolol (*PRO*), with a constant concentration of epinephrine-bound receptors. This experiment is representative of five experiments.

In the presence of the highest propranolol concentration used, the 1-min cAMP accumulation was reduced to less than 50% of control. The same concentration of propranolol had no effect on 5 μM prostaglandin E_1-stimulated cAMP accumulation (data not shown).

This result shows that a constant epinephrine occupancy of receptors does not lead to a constant rate of adenylate cyclase activation when the epinephrine is confined to a small fraction of the receptors. This constitutes a demonstration that the predictions of the collision coupling model break down under these experimental conditions.

It can be noted that the conclusion drawn from the experiment in Fig. 1 does not depend on precise knowledge of the dissociation constants. The fractional occupancy of receptors by epinephrine is given by

$$[A]/K_{d_A}/(1 + [A]/K_{d_A} + [B]/K_{d_B})$$

(from Experimental Procedures), where A is epinephrine and B is propranolol. Because after the first experimental point (no propranolol) the term $[B]/K_{d_B}$ is always larger than either $[A]/K_{d_A}$ or unity, the expression is approximated by

$$([A]/K_{d_A})/([B]/K_{d_B})$$

Thus, although a poor estimate for K_{d_A} would lead to a poor estimate for epinephrine occupancy, it would not lead to large changes in occupancy from one experimental point to the next. The experimental design required constancy of receptor occupation by epinephrine but does not require a specific particular value for occupancy. In contrast, a poor estimate for K_{d_B}, or combinations of errors in K_{d_A} and K_{d_B}, could lead to poor estimates for or changes in epinephrine occupancy but only in ways that are inconsistent with the data. For instance, an error in K_{d_B} could lead to dramatic differences in epinephrine occupancy between the control point (no propranolol) and the first point in which epinephrine and propranolol were combined, but no difference in cAMP accumulation is observed between these two points. Thus, the general conclusion from the experiment, that cyclase activation is not proportional to receptor occupancy under all circumstances, holds irrespective of the actual value for occupancy and holds even if the K_d values were different from those used for calculation of the concentrations.

Receptor participation in the presence of a rapidly dissociating antagonist, metoprolol. A second series of experiments was conducted with the β-adrenergic antagonist metoprolol. Metoprolol is a low affinity antagonist ($K_d \simeq 240$ nM), compared with propranolol, and it is, therefore, expected to have a much shorter bound-complex $t_{1/2}$ than propranolol (8, 9). High occupancy by metoprolol with constant occupancy by epinephrine can be achieved in the manner used in the propranolol experiment. Because of its shorter bound half-life, however, metoprolol would be far less effective at limiting the number of receptors accessible to epinephrine during the 1-min assay. Thus, it was predicted that use of metoprolol as a receptor-blocking agent would *not* reduce cyclase activation to the extent observed when using a high concentration of propranolol. As predicted, metoprolol had no effect on the ability of the fixed concentration of epinephrine-bound receptors to activate adenylate cyclase (Fig. 2).

Comparison of the effects of propranolol and metoprolol on k_a. From the data shown in Figs. 1 and 2, the corresponding relative changes in the rate of G protein/adenylate

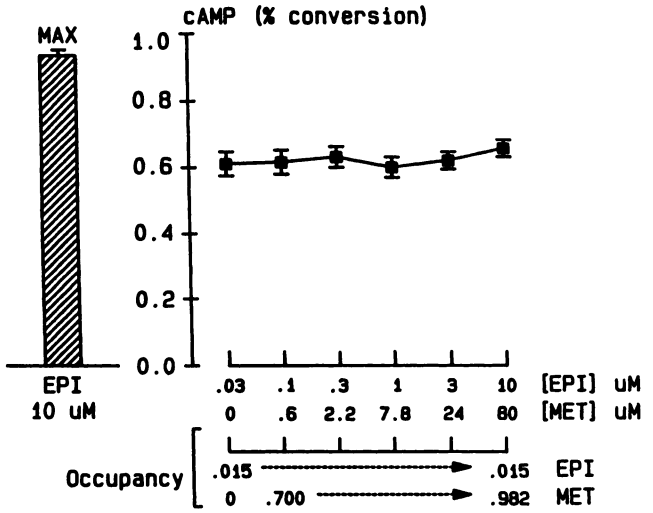

Fig. 2. cAMP accumulation (1-min percent conversion; average of triplicates ± standard error) stimulated by epinephrine (*EPI*) in the presence of varying concentrations of metoprolol (*MET*) with a constant concentration of epinephrine-bound receptors. This experiment is one of two experiments.

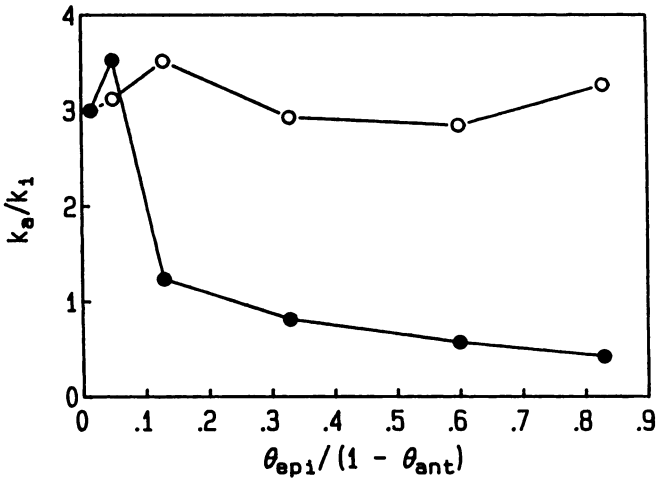

Fig. 3. Ratio of G protein/adenylate cyclase activation rate constant (k_a) to inactivation rate constant (k_i), calculated from the data in Figs. 1 and 2, versus the fraction of nonantagonist time occupied by epinephrine ($\theta_{epinephrine}/(1 - \theta_{antagonist})$). In both the propranolol and metoprolol experiments, the fraction of time not occupied by antagonist is increasingly filled by epinephrine as both epinephrine and antagonist concentrations are increased. In the propranolol experiment, the rate constant for activation decreases to an apparent limit, which is approximately 20% of control.

cyclase activation can be calculated as described in Experimental Procedures. Calculated ratios of the rate constants for G protein/adenylate cyclase activation and inactivation (k_a/k_i) for the data shown in Figs. 1 and 2 are shown in Fig. 3. For the propranolol experiment, the approximate 50% decrease in cAMP accumulation is equivalent to an 80% reduction in the rate of cyclase activation; in contrast, the presence of metoprolol had no effect on k_a. Because in both cases the concentration of epinephrine-bound receptors is the same, the difference in effects on k_a is attributable to the involvement of fewer epinephrine-bound receptors in the propranolol experiment.

Simulation of receptor occupancy: relationship of occupancy to k_a. The experiments using propranolol as described led to a reduction in the number of epinephrine-bound receptors participating in the coupling to adenylate cyclase. The difference between control (no propranolol) and high propranolol experiments can be visualized by a simulation of receptor occupancy, as shown in Fig. 4. In this figure, a simulation of the bound/unbound status of individual receptors is shown as a function of time. The control experiment had a receptor occupancy of 0.015 (or 1.5%), distributed among the 2000 receptors of the cell, of which an average of 30 were occupied at any one time, each individual receptor being occupied 1.5% of the time (Fig. 4A). With the highest concentration of propranolol added, the situation approached one in which 30 individual receptors were occupied 100% of the time (Fig. 4B). As can be seen, the addition of propranolol under this protocol reduced the number of individual receptors interacting with epinephrine per unit time, although the concentration of bound receptors cell-wide remains identical. Over the range of propranolol concentrations used in the experiment, the occupancy (which is constant) was progressively changed from the condition represented in Fig. 4A (in which switching of occupancy takes place among the entire population of receptors) to that in Fig. 4B (in which individual receptors are effectively occupied for longer periods of time). Using metoprolol, this could not occur because of the high (compared with propranolol) binding frequency of metoprolol, as depicted in Fig. 4C. In the propranolol experiments, it is apparent that the mobility of the receptors will determine the maximum cyclase activation rate, and the results indicate that the mobility of receptors is insufficient to account for activation in the normal (no antagonism) dose-response curve.

It has been observed by others (9, 10) that the dissociation rate for weakly binding agonists and antagonists of the β-adrenergic receptor is very fast, and the dissociation rate has not been measured for epinephrine or metoprolol. Here we have used rough estimates of the dissociation rates, assuming that the difference between dissociation constants is assignable to differences in dissociation rates rather than association rates [and we have assumed association rates on the order of 10^8/M/min (9–11)]. In the simulations shown in Fig. 4, the precise values for the dissociation constants are not crucial to the demonstration of the effects on occupancy of the experimental conditions; rather, the important factor is that the dissociation rates for both epinephrine and metoprolol are rapid compared with that for propranolol. In these simulations, we have used a $t_{1/2}$ of 2.5 min for the propranolol-receptor complex, measured in our laboratory. Measurements of the half-life of the propranolol-β-adrenergic receptor complex have shown it to be relatively long (9, 10, 12). Using a different S49 cell clone, others have estimated a $t_{1/2}$ of 0.7 min (12). Using this value of $t_{1/2}$ would alter the simulation in its details, but it would make no difference to whether receptors can effectively be "insulated" against agonist occupancy for periods of time that are long compared with the half-life of epinephrine occupancy and that result in prolonged occupancy by epinephrine of individual receptors.

Comparison of experiment to an estimation for mobility-limited activation. A theoretical maximum adenylate cyclase activation rate can be calculated for the mobility-limited case on the basis of the probability of encounter between receptor and cyclase, for a given number of occupied receptors, using a diffusion coefficient to characterize receptor mobility and assuming an efficiency of unity, i.e., every encounter leads

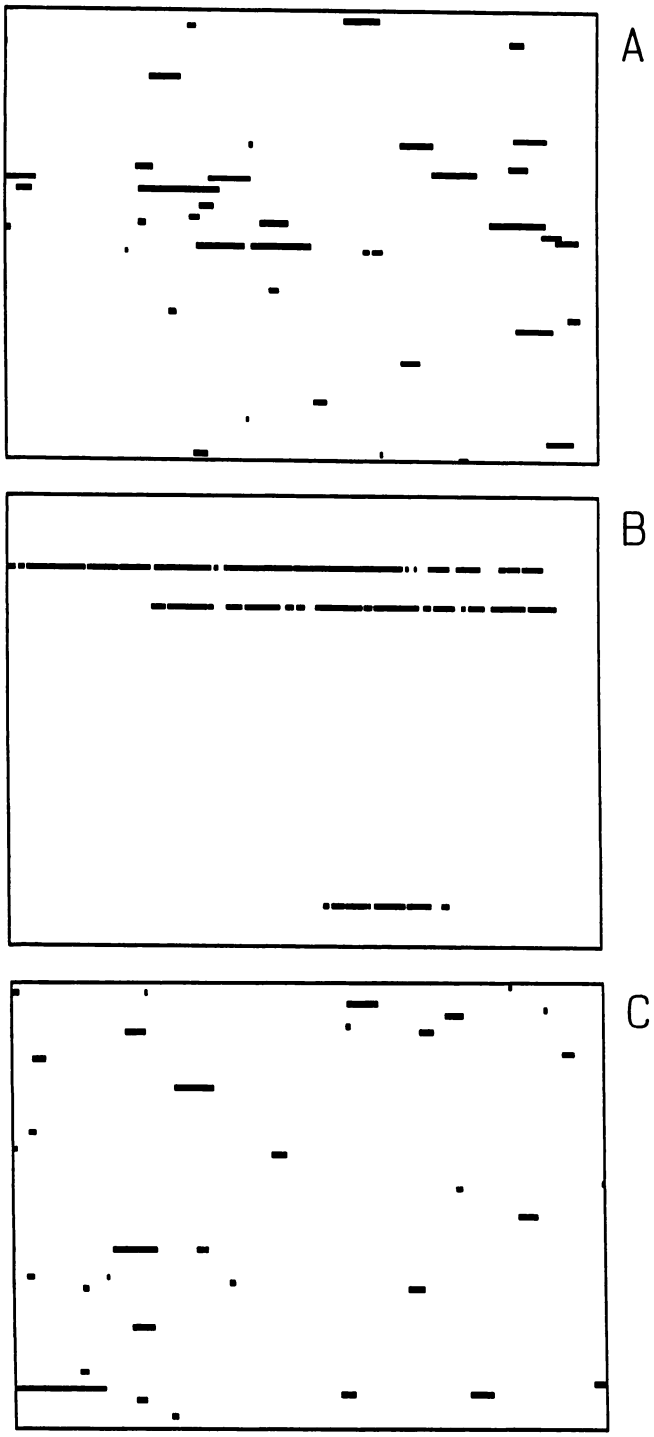

Fig. 4. Simulations of individual receptor binding to epinephrine for three conditions: 30 nM epinephrine (control) (A); 10 μM epinephrine and 215 nM propranolol (B); and 10 μM epinephrine and 80 μM metoprolol (C). Each receptor is represented as a *track* in the figure (80 *tracks* total) moving through time (0.1 min) from *left* to *right*; *dark* indicates bound to epinephrine, *light* indicates unbound or bound to antagonist. In all three cases, the average occupancy by epinephrine is identical. The simulations (corresponding to conditions for points in the experiments shown in Figs. 1 and 2) were made using the following constants: epinephrine, $k_1 = 1.4 \cdot 10^8$/M/min, $K_d = 2$ μM; propranolol, $k_1 = 4 \cdot 10^8$/M/min, $K_d =$ 650 pM; and metoprolol, $k_1 = 4 \cdot 10^8$/M/min (assumed to be equal to propranolol), $K_d = 240$ nM. Simulation step size = 0.0002 min.

to activation. It is important in this context to distinguish between "encounter" and "collision." In the liquid phase in general, but especially in the confinement of the two-dimensional cell membrane, two molecules that collide with each other do so a large number of times before diffusing apart; we call this series of collisions an encounter. The approach to calculating a limit to mobility-limited activation is based on the fact that an adenylate cyclase that does not encounter a receptor-agonist complex cannot be activated. The probability of an encounter is not greater than the probability of spatial coincidence of receptor and cyclase on the cell surface, which can be calculated given the mobility of the receptor in the membrane.

A theoretical dose-response curve for the mobility-limited case can be derived with the assumption that switching of agonist from receptor to receptor cannot occur. An equation is derived in the Appendix that relates the cyclase activation rate to the fraction of the cellular surface that is visited per unit time by n receptor-agonist complexes. A theoretical dose-response curve is generated from the equation by calculating the activation rate for $n = 0$ to $n = N$ receptors, where N is the maximum number of the β-adrenergic receptors in the cell. Thus, when the receptor occupancy is θ, we assume that $n = N\theta$ receptors are occupied by agonist all of the time. The remaining receptors are assumed to have no bound agonist at any time and, hence, cannot participate in the activation. The S49 variant we have been using averages $N = 2000$ receptors/cell. For a given concentration of agonist leading to occupancy θ, the number of receptor-agonist complexes is given by $n = 2000 \times \theta$.

As demonstrated in Fig. 4, epinephrine switching between receptors is greatly reduced at high propranolol concentrations. At the highest concentrations used in those experiments, there is very little time between successive epinephrine bindings to a single receptor. Thus, the adenylate cyclase activation rate should approximate, under those conditions, one with no contribution from agonist switching. Fig. 3 illustrates that the rate with which a given number of epinephrine-receptor complexes activate adenylate cyclase is reduced to about 20% of control when switching is effectively prevented. This is equivalent to an increase in the EC_{50} from 10 nM, determined with all the receptors freely available (the normal epinephrine dose-response curve), to approximately 60 nM. The equation developed in the Appendix relates the adenylate cyclase activity in terms of the diffusion coefficient, cell size, and receptor number for the mobility-limited case. Cell size and receptor number are known; the diffusion coefficient necessary to account for the EC_{50} of 60 nM for the mobility-limited case can, therefore, be calculated from the model equations. A mobility-limited dose-response curve calculated by this method is compared with the "normal" epinephrine dose-response curve in Fig. 5.

Calculation of a mobility-limited curve such as shown in Fig. 5 required a number of input values taken as constants: $K_d =$ 2 μM for epinephrine; the inactivation rate constant for adenylate cyclase, $k_i = 1.4$/min; the total number of S49 cell β-adrenergic receptors, $N = 2000$; and the diameter of the S49 cell, $d = 9$ μm. The dependence of the calculated curve on these parameters is illustrated in Fig. 6.

Because the diffusion coefficient (D) of β-adrenergic receptors has not been measured directly, we back-calculated D from Eq. 2 in the Appendix. The mobility-limited curve has an EC_{50}

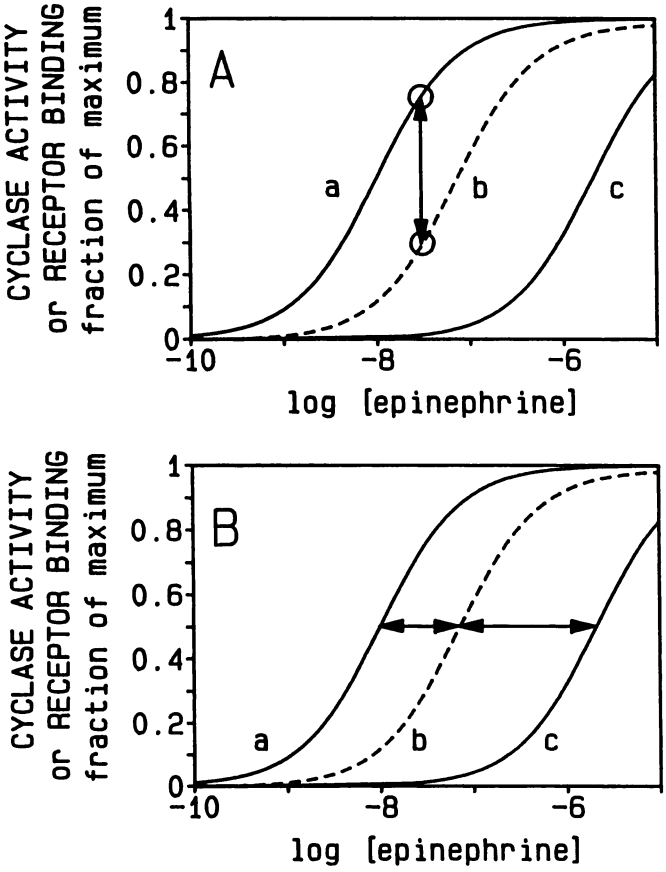

Fig. 5. Comparison of binding, response, and calculated mobility-limited response curves for epinephrine-stimulated S49 cells. A, Experimental cyclase activity "normal" dose-response curve characterized by EC_{50} = 10 nM (*a*) compared with theoretical curve calculated for receptor mobility-limited case characterized by EC_{50} = 60 nM (*b*). Approximately 50% of the total activity observed at 30 nM epinephrine in the normal dose-response curve is attributable to switching of epinephrine among the entire receptor population, as shown by the *arrow* indicating the range of the data in Fig. 1 for epinephrine occupancy equivalent to 30 nM epinephrine. The binding curve characterized by K_d = 2 μM is also shown (*c*). B, The larger fraction of the total separation (pharmacological shift ratio) between response and binding curves is largely attributable to the receptor mobility.

of approximately 60 nM; calculating backward from Eq. 2 gives the value D = 0.008 μm^2/sec. This is greater than D for β-adrenergic receptors in turkey erythrocyte membranes, 0.004 μm^2/sec, reported by Hanski *et al.* (13), which was back-calculated by another means from their cyclase activation data. Both values for D are somewhat less than the value D = 0.05 μm^2/sec measured for insulin and epidermal growth factor receptors (14). As in the work of Hanski *et al.* (13), this is due in part to the generosity of our assumptions for calculating the rate of activation, in which spatial proximity is equated to an encounter with a 100% efficiency of activation; a more rigorous treatment, diminishing encounter frequency and efficiency of activation, would require a higher value for D to account for the same extent of cyclase activation.

This derivation is an extension of a calculation made by Swillens and Dumont (15), which showed that mobility of receptors would not be a limitation in activation in erythrocytes (d = 11 μm) if receptors and cyclase are in equimolar amounts (1000/cell), when the full number (N) of the receptors are occupied 100% of the time and $D \simeq 0.09$ μm^2/sec. As can be seen from our calculated activity curve, the present derivation asserts that this would also be true for the S49 cell using any reasonable value for D, because at high occupancy the theoretical diffusion-limited curve closely approximates the experimental curve.

Effect of preaddition of antagonist. Careful attention is necessary in the addition of agonist and antagonist for the measurements of adenylate cyclase activity, because of the differences in time required for each to reach binding equilibrium. Because the agonist is generally present at a much greater concentration than the antagonist, simultaneous addition of the final concentrations of each at the beginning of the assay would give greater than equilibrium occupancy by agonist at early times. This would lead to a greater-than-steady state activation of adenylate cyclase, which would be especially troublesome in 1-min incubations used in these studies. Preaddition of the antagonist in order to allow steady state antagonist occupancy before the addition of agonist eliminates this possibility. This option was used in the experiments shown in Figs. 1 and 2. However, preaddition of the full final amount of antagonist (calculated on the basis of the presence of agonist) potentially leads to an underestimate of adenylate cyclase activity, because the rate at which agonist can bind depends largely on the dissociation rate for the antagonist. A third option is the preaddition of a concentration of antagonist calculated to occupy the fraction of receptors it is to occupy in the presence of agonist during the assay, followed by an amount calculated to compensate for the displacement by agonist upon addition of agonist ("split" addition). This third option virtually eliminates both problems of agonist overshoot or undershoot. Theoretical receptor occupancy by epinephrine as a function of time for these various options and for the experimental conditions can be calculated using an analytical solution to the equations for competitive binding, as described by Motulsky and Mahan (12), using the constants given in Fig. 4. Compared with the control point in these experiments (30 nM epinephrine alone), a split addition of propranolol (36 nM propranolol at t = −6 min, followed by additions to give 215 nM propranolol and 10 μM epinephrine at t = 0) leads to epinephrine occupancy that is >95% of control 1 sec after epinephrine addition. Preaddition (215 nM propranolol at t = −6 min, followed by 10 μM epinephrine at t = 0) leads to epinephrine occupancy that is >90% of control 10 sec after epinephrine addition. In order to test the possibility that the observed differences in cAMP accumulation were due in part to these slight differences in the time to steady state epinephrine occupancy, experiments were conducted to compare preaddition with split addition (with final concentrations of epinephrine = 10 μM, propranolol = 215 nM, equivalent to the last point in Fig. 1), which resulted in no measurable difference in 1-min cAMP accumulation (data not shown).

Discussion

The experiments were designed to measure the adenylate cyclase activation rate for a constant concentration of epinephrine-bound receptors, while varying the number of receptors involved in the activation process. The results demonstrate a significant difference in activation rate when occupancy of receptors by epinephrine is restricted to a small number of receptors rather than being distributed over the entire population of receptors. Thus, the adenylate cyclase activation rate is

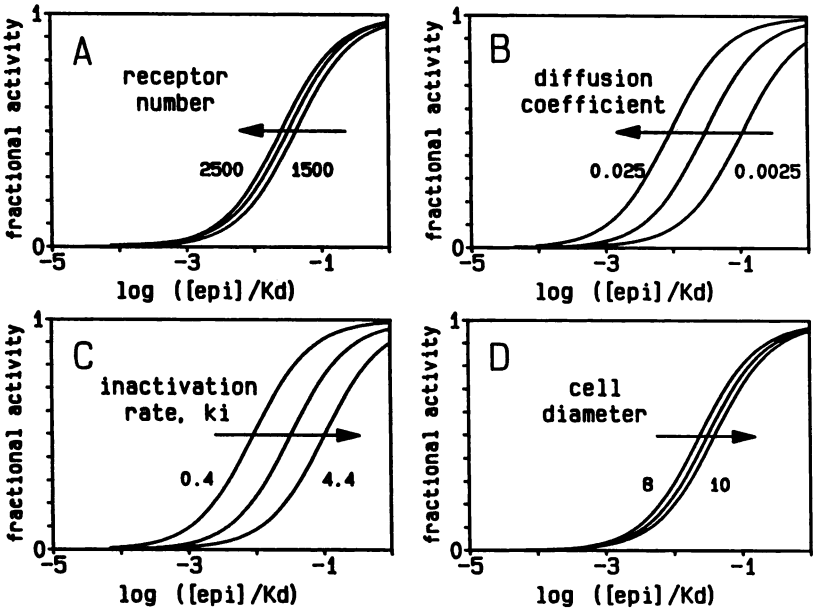

Fig. 6. Calculated mobility-limited dose-response curves for epinephrine stimulation of S49 cell adenylate cyclase, using different values of parameters in the model for diffusion-limited activation: receptor number, N (A); diffusion coefficient, D (μm^2/sec) (B); rate constant for cyclase inactivation, k_i (1/min) (C); cell diameter, d (μm) (D). The *center curve* in each *panel* uses the same values as in Fig. 5: $N = 2000$, $D = 0.008$ μm^2/sec, $k_i = 1.4$/min, and $d = 9$ μm.

not proportional to the concentration of agonist-bound receptors in the case where epinephrine is the agonist. This is an indication that receptor mobility by itself is insufficient to account for the wide separation (pharmacological shift ratio) between epinephrine binding and the epinephrine response curves. Epinephrine appears to overcome the receptor mobility limitation by a high frequency of binding.

The detection of the agonist-switching effect depends on a high efficiency of activation during a receptor-cyclase encounter; were the probability of activation during a receptor-cyclase encounter low, there would then be no advantage or observable effect to accompany high binding frequency. Correspondingly, this effect might not be detectable under conditions where that efficiency is significantly reduced. There exists a clear advantage, then, of the use of intact cell preparations over broken cell preparations. Because the number of receptors in each unit of the system (i.e., in each cell) is large, there is little risk that an effective reduction in receptor number might lead to the situation where cyclase has no access to receptors. This is not necessarily true in broken cell preparations, where the sizes of the vesicles that bear the cyclase are generally unknown and where there may be only a few receptors per vesicle even in the control preparations. Quite apart from vesicle size, possible changes in the viscosity of the membrane after vesiculation might have dominating effects on the activation parameters.

The disadvantages of intact cell use are that adenylate cyclase activity measurements are influenced by ongoing hydrolysis of the cAMP and by desensitization of the adenylate cyclase during the course of the assay. In the present case, these are minimized by the use of short assay times, during which the extents of both hydrolysis and desensitization are small. In S49 wild type cells, the cAMP accumulation during the first minute of stimulation and the initial rate of cAMP synthesis differ by only a few percent (1). The accumulation data are, therefore, a good estimate for the rate of synthesis of cAMP by adenylate cyclase. Moreover, it should be noted that the main conclusion of these studies, that rapid dissociation and association of agonist from receptors is important, is not dependent on a strict linear relationship between rate of cAMP synthesis and cAMP accumulation during 1 min. The demonstration that accumulation is less in 1 min is sufficient to show that cAMP synthesis, and therefore the cyclase activation rate, has declined.

Experimental evidence for the role of epinephrine binding frequency forms an extension of the results of Hanski *et al.* (13), which dealt explicitly with the relationship between receptor mobility and the collision coupling model. In their work, the nonhydrolyzable GTP analogue guanosine 5′-(β,γ-imido)triphoshate was used with epinephrine-stimulated turkey erythrocyte membranes in order to examine the effects of membrane fluidity on the rate of activation of adenylate cyclase. The cyclase activation rate was shown to increase linearly (up to 20-fold) as a function of the membrane fluidity, using 0.1 mM epinephrine (for which receptor occupancy is nearly 100%). This effect of membrane fluidity on the activation rate is consistent with the collision coupling model and is an indication that the activation process is diffusion controlled at high concentrations of epinephrine. Our results indicate, however, that this is not true for epinephrine stimulation over the entire range of the dose-response curve. When receptors are less than 100% occupied, the binding frequency of epinephrine overcomes the mobility limitation.

The data are not incompatible with the concept that receptors (and cyclase) diffuse freely in the membrane nor with the idea that one receptor can, within a short space of time, activate a large number of cyclase moieties. Rather, these presumptions are strengthened by the data. The data challenge only the assumption that diffusion alone can account for all of the pharmacological shift ratio of epinephrine. At the highest concentrations of epinephrine and propranolol shown in Fig. 1, only 1.5% of about 2000 receptors are not bound by propranolol at any one time and most of the receptors are not available for epinephrine binding at any time during the assay. Nevertheless, about 30% of the adenylate cyclase is activated under this circumstance. This implies that 30 receptors can cover a very significant fraction of the cell surface during a time period comparable to the inactivation cycle of adenylate cyclase.

An alternative approach to examining the contribution of

binding frequency would be to irreversibly block some fraction of the receptors by pretreatment with an irreversibly binding β-adrenergic antagonist (16). In principle, using these agents one could measure occupancy directly rather than rely on calculations of probabilities that rely on values for K_d. There are, however, a number of drawbacks in practice that limit the usefulness of such an approach. First, it would be correspondingly more difficult to measure with any accuracy the number of remaining receptors when the number is brought down to such low numbers as would be necessary for these experiments. Second, it would prove difficult using such agents to manipulate the system so as to consistently bring the receptor level to a fixed prescribed level. The advantage of our technique is that we can achieve the effect of reduced receptor number by manipulation (without, in fact, requiring unreasonable precision in K_d values) of antagonist and agonist concentrations. Moreover, the approach used here also eliminates the need for potentially damaging additional steps in cell handling before measurements in cAMP accumulation are made.

Departure from the predictions of the collision coupling model only becomes large when the effective number of receptors is reduced to less than 10% of its normal value. However, this situation is not unknown in clinical applications. Propranolol is typically given to patients at doses that correspond to mean body concentrations of over 500 nM, which is sufficient to occupy over 99% of β-adrenergic receptors of the type studied here. Under these circumstances, reduction in response to agonist will be a great deal larger than that predicted by the Cheng-Prusoff equation (17). The results obtained with metoprolol indicate a potentially important clinical difference as a result of its high binding frequency relative to propranolol; a metoprolol-antagonized system would retain greater responsiveness to transient changes in agonist concentration. A response-producing change in agonist concentration in the presence of propranolol would need to be comparatively prolonged and persistent.

The results explain why epinephrine has such a high pharmacological shift ratio in spite of its relatively low affinity for the β-adrenergic receptor. Presumably, the conformational state induced in the β-adrenergic receptor by other agonists (e.g., isoproterenol) is equally efficient at activating adenylate cyclase (efficiency being the fraction of collisions that are successful at activation). Yet the short half-life of the epinephrine-β-adrenergic complex (high binding frequency of epinephrine) renders epinephrine more efficient at covering the cell surface without wasting collisions with cyclase that is already active. The association rate constant for a number of β-adrenergic receptor ligands has been estimated to be on the order of 10^8/M/min (9–11). Given a K_d of 2 μM for epinephrine in this system, the dissociation rate constant is 200/min, which gives a $t_{1/2}$ for the bound receptor complex of 0.2 sec. Concomitantly, the time required for activation during an encounter between receptor and cyclase appears to be small, although the time the receptor and cyclase remain in potentially interactive proximity may be quite large.

In summary, the results demonstrate that receptor mobility is not sufficient by itself to account for epinephrine-stimulated cyclase activity along the entire response curve in the intact S49 cell. Receptor mobility is nonetheless sufficient to account for the majority of the separation between the binding and response curves, i.e., of a 200-fold separation between EC_{50} and K_d for epinephrine, receptor mobility accounts for approximately 30-fold of the leftward shift of the activation curve relative to the binding curve. Epinephrine binding frequency appears to account for the remainder (a factor of 6) of the separation. Correspondingly, the phenomenon of switching, given the high epinephrine binding frequency, contributes at least 50% to the observed activity for epinephrine stimulation at concentrations near the EC_{50}. We, therefore, conclude that a significant portion (>80%) of the rate of adenylate cyclase activation by epinephrine may be attributed to switching among β-adrenergic receptors in this region of the response curve.

A complete model of adenylate cyclase activation will need to take into account not only the agonist dissociation constant but also the binding frequency, agonist efficiency, and receptor mobility. Understanding the relationships between these parameters and cyclase activity may lend some insight into mechanisms of desensitization of the cAMP-generating system that involve β-adrenergic receptors directly.

Acknowledgments

The authors wish to thank Derek S. Ng Tang for expert technical assistance.

Appendix: Mathematical Model of Activation for the Receptor Diffusion-Limited Case

Here we derive an expression for the extent of adenylate cyclase activation for the hypothetical case of n receptors that are 100% occupied by a completely efficient agonist.

Let receptor mobility be characterized by a diffusion coefficient D. The average radial distance l that a single receptor moves away from its initial position during time τ is given by:

$$l = \sqrt{4D\tau}$$

For sufficiently small τ, the random motion of the receptor may be treated as having been confined to an area defined by radius l, which defines a maximum radius of interaction for the receptor during time τ. This radius defines an area (a) covered by a receptor in time τ:

$$a = \pi l^2 = 4\pi D\tau$$

From the standpoint of a cyclase molecule, the probability of not encountering this receptor (P_0) is at least as the great as the fraction of the available surface area (the cell surface area, a_{cell}) that is not covered by the receptor:

$$P_0 > (a_{cell} - a)/a_{cell}$$
$$= (\pi d^2 - 4\pi D\tau)/(\pi d^2)$$
$$= (1 - 4D\tau/d^2)$$

for a cell of diameter d. If there are n such receptors occupied, the probability of not encountering any one of n occupied receptors is given by:

$$P_0 > (1 - 4D\tau/d^2)^n$$

such that the probability (P_1) of an encounter is:

$$P_1 < 1 - P_0 = 1 - (1 - 4D\tau/d^2)^n$$

If we allow the agonist-bound receptor to be maximally efficient at cyclase activation, then the probability of an encounter between agonist-bound receptor and cyclase is equal to the probability of activation of cyclase. This probability may be converted to a (maximum) rate constant for activation (k_a) by:

$$P_1 = 1 - e^{-k_a\tau}$$

$$k_a = -1/\tau \ln(P_1 - 1) = -1/\tau \ln(P_0)$$

$$= -1/\tau \ln(1 - 4D\tau/d^2)^n$$

$$= -n/\tau \ln(1 - 4D\tau/d^2)$$

A hypothetical dose-response curve for agonist A can be calculated using this expression by letting $n = N\theta$, where N is the total number of receptors on a cell and θ is the fractional occupancy of the receptors, which is a function of $[A]$:

$$n = N\theta = N[A]/([A] + K_d)$$

such that

$$k_a = -(N\theta/\tau)\ln(1 - 4D\tau/d^2) \quad (1)$$

$$k_a = -(N/\tau)[A]/([A] + K_d)\ln(1 - 4D\tau/d^2)$$

Fractional activation (f) of adenylate cyclase is then calculated as a mass balance between rates of activation and inactivation, using k_a from Eq. 1:

$$f = k_a/(k_a + k_i) \quad (2)$$

which can be compared with the experimental value for f:

$$f = [A]/([A] + EC_{50})$$

where EC_{50} = 10 nM for the normal dose-response curve. Note that the equation for f, including substitutions for k_a, is a hyperbola, as are the usual equations that describe activation and binding.

References

1. Barber, R. Discrimination between intact cell desensitization and agonist affinity changes. *Mol. Cell. Endocrinol.* **46**:263–270 (1986).
2. Macfarlane, D. Bidirectional collision coupling in the regulation of adenylate cyclase. *Mol. Pharmacol.* **22**:580–588 (1982).
3. Tolkovsky, A., and A. Levitzki. Mode of coupling between the β-adrenergic receptor and adenylate cyclase in turkey erythrocyte membranes. *Biochemistry* **17**:3795–3810 (1978).
4. Tolkovsky, A., S. Braun, and A. Levitzki. Kinetics of interaction between β-receptors, GTP protein, and the catalytic unit of turkey erythrocyte adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **79**:213–217 (1982).
5. Swillens, S. Modulation of catecholamine activation of adenylate cyclase by the number of active β-adrenergic receptors: theoretical considerations on the role of receptor diffusion in the cell membrane. *J. Cyclic Nucleotide Res.* **8**:71–82 (1982).
6. Barber, R. Forskolin binding to intact S49 lymphoma cells. *Second Messengers Phosphoproteins* **12**:59–71 (1988).
7. Cassel, D., and Z. Selinger. Catecholamine-stimulated GTPase activity in turkey erythrocyte membranes. *Biochim. Biophys. Acta* **452**:538–551 (1976).
8. Toews, M., T. Harden, and J. Perkins. High affinity binding of agonists to β-adrenergic receptors on intact cells. *Proc. Natl. Acad. Sci. USA* **80**:3553–3557 (1983).
9. Insel, P., L. Mahan, H. Motulsky, L. Stoolman, and A. Koachman. Time dependent decreases in affinity of agonists for β-adrenergic receptors of intact S49 lymphoma cells. *J. Biol. Chem.* **258**:13597–13605 (1983).
10. Contreras, M., B. Wolfe, and P. Molinoff. Kinetic analysis of the interactions of agonists and antagonists with β-adrenergic receptors. *J. Pharmacol. Exp. Ther.* **239**:136–143 (1986).
11. Meuller, H., H. Motulsky, and L. Sklar. The potency and kinetics of the β-adrenergic receptors on human neutrophils. *Mol. Pharmacol.* **34**:347–353 (1988).
12. Motulsky, H., and L. Mahan. The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol. Pharmacol.* **25**:1–9 (1984).
13. Hanski, E., G. Rimon, and A. Levitzki. Adenylate cyclase activation by the β-adrenergic receptors as a diffusion-controlled process. *Biochemistry* **18**:846–853 (1979).
14. Schechter, Y., J. Schlessinger, S. Jacobs, K. Chang, and P. Cuatrecasas. Fluorescent labeling of hormone receptors in viable cells. *Proc. Natl. Acad. Sci. USA* **75**:2135–2139 (1978).
15. Swillens, S., and J. Dumont. A unifying model of current concepts and data of adenylate cyclase activation by β-adrenergic agonists. *Life Sci.* **27**:1013–1028 (1980).
16. Pitha, J., J. Zjawiony, R. Nasrin, R. Lefkowitz, and M. Caron. Potent β-adrenergic antagonist possessing chemically reactive group. *Life Sci.* **27**:1791–1798 (1980).
17. Cheng, Y., and W. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).

Send reprint requests to: Douglas Stickle, Laboratories of Cyclic Nucleotide Research, Graduate School of Biomedical Sciences, University of Texas Health Science Center, P.O. Box 20334, Astrodome Station, Houston, TX 77225.