Biochemical Pharmacology, Vol. 42, No. 5, pp. 1069–1077, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

ESTIMATION OF THE KINETIC CONSTANTS FOR BINDING OF EPINEPHRINE TO β-ADRENERGIC RECEPTORS OF THE S49 CELL

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(*Received* 25 *February* 1991; *accepted* 12 *April* 1991)

Abstract—The dissociation constant (K_d) for the binding of epinephrine to β-adrenergic receptors of the S49 cell is 2 μM, which is the ratio of the rate constants for dissociation (k_{off}) and association (k_{on}), $K_d = k_{off}/k_{on}$. Although the K_d is known by direct measurement, the individual rate constants k_{on} and k_{off} are unknown since they are both too large to be measured by conventional experimental methods. We present here an analysis in which a minimum value for these constants is calculated. The analysis uses a "transiently private receptor" model for coupling of receptors to G protein/adenylate cyclase that is based on the limits prescribed by the known empirical relationship between the β-adrenergic receptor occupancy by epinephrine (characterized by K_d) and their coupling to adenylate cyclase (characterized by EC_{50} = 10 nM) as a function of epinephrine concentration. The model makes only the assumption (based on previous evidence) that a receptor cannot activate more than one cyclase during the course of one cycle of binding and unbinding of an epinephrine molecule. In such a model, and with such a large separation between the K_d and the EC_{50}, the rate of G protein/adenylate cyclase activation by epinephrine-bound receptors can be related to the frequency of receptor binding at low concentrations of epinephrine, from which minimum values for the rate constant for association can be derived. This gives the estimates $k_{on} > 10^8$/M/min and $k_{off} > 280$/min at 37°. The on-rate constant is comparable to the on-rate constants that have been measured for other β-adrenergic receptor ligands. This result supports the transiently private receptor coupling model as an explanation for the previously observed contribution of the frequency of epinephrine binding to the rate of activation of adenylate cyclase in the S49 cell.

The dissociation constant K_d represents the ratio of the first-order dissociation rate constant (k_{off}) and the second-order association rate constant (k_{on}) for the reversible binding of ligand (L) to cell surface receptors (R = unbound, R* = bound):

$$L + R \rightarrow R^*$$

bimolecular rate constant k_{on} (≡ 1/M/min)

$$R^* \rightarrow L + R$$

monomolecular rate constant k_{off} (≡ 1/min)

$$K_d = k_{off}/k_{on} \ (\equiv M).$$

The K_d can be thought of in at least three mathematically equivalent ways: it is the concentration of ligand for which at steady state: (1) the fraction of bound receptors is equal to the fraction of unbound receptors (= 0.5); (2) any given receptor is occupied 50% of the time; or (3) the probability is 0.5 that any given receptor is occupied at any given instant.

The K_d for epinephrine is approximately 2 μM for the β-adrenergic receptors of the intact S49 cell [1], obtained from data of 1-min displacement of labeled antagonist (^{125}I-cyanopindolol) as a function of unlabeled epinephrine concentration using the nonequilibrium technique of Toews *et al.* [2]. The individual rate constants k_{on} and k_{off} for epinephrine remain undetermined, however, because of the difficulty in direct experimental measurement due to their apparently large magnitude. Methods for the direct measurement of rate constants involve measurement of the rates of change in cell-associated radiolabeled ligand tracers as a function of ligand concentration [3, 4]. From the association and dissociation reactions given above, the time course for the change in the fraction of receptors bound to ligand ($\theta = R^*/R_{total}$) upon changes in ligand (L) concentration is given by:

$$\theta(t) = \theta_{ss} - [\theta_{ss} - \theta(0)]\, e^{-(k_{on}[L] - k_{off})t} \quad (1)$$

where $\theta(0)$ is the initial value for θ at $t = 0$, and θ_{ss} is the steady-state value for θ. For epinephrine binding the change to a new steady state after a change in epinephrine concentration is too rapid for the measurement of any intermediate points from which the binding rate constants could be derived. Thus, the rate constants for epinephrine binding are apparently so large that they cannot be determined by any method which relies on resolution of a time course. This limitation allows, however, one estimate of a minimum value for k_{on} for epinephrine to be made by defining an arbitrary time for resolution of experimental binding data. For instance, the value $k_{on} > 1 \times 10^7$/M/min leads to a system for which the maximum half-time for binding (as [L]→0, $t_{1/2} \rightarrow \ln 2/(k_{off})$) is approximately 2 sec for K_d = 2 μM.

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Another approach to determining the rate constants for binding involves the relationship of binding to a functional effect of the bound state of the receptor [5]. Occupancy by epinephrine of the β-adrenergic receptor leads to the activation of GTP-binding protein/adenylate cyclase and the production of cyclic adenosine monophosphate (cAMP). The important aspect of the relationship between binding and response for activation of adenylate cyclase in epinephrine-stimulated S49 cells is that there is a wide separation between the K_d and the EC_{50}, where EC_{50} is the epinephrine concentration which gives a half-maximal rate of cAMP formation. For this system $K_d = 200\,EC_{50}$, i.e. receptors need to be occupied by hormone only a small fraction of the time in order to promote near-maximal activation.

A priori, this relationship between receptor binding and response can be explained by a number of very different schemes. For instance, a "private" receptor model, in which the receptor and GTP-binding protein are precoupled, could give such a relationship. If the rate of activation upon agonist binding is rapid, but the rate of inactivation (GTPase activity) is slow and independent of receptor activity, then high activity can be maintained by relatively low occupancy of the receptor (e.g. periodic episodes of occupancy of short duration). In such a model the rate of adenylate cyclase activation is proportional to the frequency of the binding of individual receptors. The private receptor model is known to be false, since irreversible blockade of receptors does not proportionally diminish adenylate cyclase response [6]. In fact, it is this relationship between receptor number and cyclase activation which led to the development of the accepted explanation for the relationship between binding and response, which is the Collision Coupling Model by Tolkovsky and Levitzki [6]. In the collision coupling model (a "promiscuous" receptor model), receptors have access to more than one G protein and act as mobile catalysts for G protein activation; the model incorporates the Cassel and Selinger model [7–9] for G protein inactivation (the GTPase activity, which is relatively slow) that is independent of receptor activity, and predicts a separation between the K_d and the EC_{50} that is necessarily large for an efficient agonist [10]. The basic tenets of the collision coupling model have been supported by a variety of types of evidence [11–14].

In its simplest form the collision coupling model assumes that the cyclase activation rate is proportional to the concentration of bound receptors [6]. That assumption makes no distinction, however, between bound-receptor concentrations comprised by a low receptor occupancy (the fraction of time a given receptor is bound) of a large number of receptors versus high occupancy of a low absolute number of receptors: for instance, is there a difference between having 30 receptors (of approximately 2000 total for the S49 cell) bound at steady state (meaning a given receptor is occupied 30/2000 of the time) and having only 30 receptors occupied continuously? The plausibility of the difference between the two cases relies, in part, on both receptor mobility and on the frequency with which an individual receptor in the first case toggles between the states of being bound and unbound. The binding frequency is, in turn, a function of the rate constants for the association and dissociation reactions between epinephrine and the β-adrenergic receptor at a given epinephrine concentration.

We have demonstrated recently that the two cases are quite different with respect to adenylate cyclase activation rates, and that there are circumstances in which the contribution of binding frequency to activation rates is apparent for epinephrine-stimulated G protein/adenylate cyclase in the S49 cell [15]. Specifically, it was shown that the rate of cyclase activation depends not only on the concentration of epinephrine-bound receptors but also on the rapidity with which that concentration is redistributed among the cell's entire receptor population. Isolation of a constant, low concentration of epinephrine-bound receptors from the entire receptor population to a limited number of receptors was shown to result in a decrease in the rate of activation of adenylate cyclase. This is evidence that receptor mobility by itself is insufficient to account for relationship between binding and response throughout the entire concentration–response curve, and an indication that some fraction of the separation between the binding curve (at low occupancy) and the response curve (in its steepest region) is attributable to the high binding frequency of epinephrine (the rate of turnover of epinephrine occupancy with respect to individual receptors). Essentially, by being bound for only a short period, any degree of occupancy in the normal binding curve is rapidly distributed among discontinuous regions of the cell surface, resulting in a greater activation rate, per occupied receptor, than would be possible were occupancy not so rapidly distributed. When occupancy of individual receptors is effectively prolonged, those receptors apparently "waste" collisions with adenylate cyclase which they have already activated. It follows from this observation that (1) individual pairs of receptor and G protein "collide" more than one time if they collide at all (a series of interactions which we have called an "encounter"); (2) the process of binding and unbinding of epinephrine from individual receptors takes place in a time frame which is significantly less than the time frame of such an encounter; and (3) the epinephrine-bound receptor is very efficient at activating cyclase during an encounter. Thus, even though one receptor has free access to many G proteins, there are aspects of the interaction between receptors and G protein that are analogous to private receptors on a relatively short time scale. Specifically, it is improbable that one receptor can activate more than one G protein during the course of one cycle of binding and unbinding of epinephrine, and the transient interaction between individual receptor/G protein occurs on a time scale which is greater than the mean lifetime of an individual epinephrine–receptor complex.

These observations suggest a means for the evaluation of the rate constants characterizing epinephrine binding. A model for receptor/G protein coupling in which the receptor is "transiently private" in its association with a single G protein, and which incorporates the known empirical relationships

between epinephrine concentration, receptor binding and adenylate cyclase activation, allows estimates for minimum values for the individual rate constants k_{on} and k_{off} for epinephrine binding and unbinding to β-adrenergic receptors to be calculated. The purpose of this paper is to describe in detail this calculation and its rationale. The model makes only the assumptions derived from the previous data described above: that it is improbable that one receptor can activate more than one G protein during the course of one cycle of binding and unbinding of epinephrine, and the transient interaction between individual receptor/G protein occurs on a time scale which is greater than the mean lifetime of an individual epinephrine–receptor complex. Below we develop and compare three variations of such a model for the calculation of the binding rate constants. For each, the results are quantitatively similar to the values that might be expected on the basis of the binding rate constants known for other β-adrenergic ligands in cases where it has been possible to measure them. It is thus demonstrated that the transiently private receptor model does not require any unusual values for the rates of binding as a plausible explanation for the effects of epinephrine binding frequency on the cyclase response. The relationship of this model to the collision coupling model of adenylate cyclase activation is discussed.

MATHEMATICAL MODELS FOR CALCULATION OF THE ASSOCIATION RATE CONSTANT

As a starting point for the development of the models relating cyclase activity to k_{on}, consider the kinetics of activation of adenylate cyclase (kinetically equivalent to the kinetics of activation of G protein [13, 16]), irrespective of any model for the coupling of receptors to cyclase. Designating the active and inactive forms A and B, respectively, then at a steady state in which cyclase is activated some fraction, f, of the time, the probability, P, of being in the active form $P(A)$ is given by

$$P(A) = f = \frac{k_a}{k_a + k_i}$$

where k_a is the rate of the activation and k_i is the rate of inactivation for an activated G protein/cyclase complex (i.e. the rate constant for GTPase activity). Thus,

$$k_a = k_i \frac{f}{(1-f)}.$$

Empirically, in the presence of an agonist L, f is given by

$$f = \frac{[L]}{[L] + EC_{50}}$$

where [L] is the ligand (agonist) concentration. Thus,

$$k_a = k_i \frac{[L]}{EC_{50}}.$$

This expression is simply an empirical relationship, to be used below, between the rate of activation and the rate of inactivation for cyclase given an agonist concentration and the EC_{50} for that agonist.

Limiting case: precoupled receptor and cyclase

Second, consider the relationship between this k_a and the agonist binding frequency in a hypothetical private receptor model in which the activation rate is completely dependent on the agonist binding frequency. A completely frequency-dependent situation would be one in which one receptor is continuously in a position to activate one adenylate cyclase to which it is permanently and privately coupled, and in which the receptor is perfectly efficient at activation upon binding (i.e. cyclase would be activated instantaneously upon binding of epinephrine to the receptor) as depicted in Fig. 1. In such case the rate of cyclase activation would be equivalent to the rate of ligand binding, which is the product of k_{on} and [L]:

$$k_a = k_{on}[L]$$

leaving

$$k_{on}[L] = k_i \frac{[L]}{EC_{50}}$$

or

$$k_{on} = \frac{k_i}{EC_{50}}.$$

The value k_{on} derived from a frequency-dependent scheme is simply a ligand binding association rate constant which would result in the proper concentration–response relationship between ligand concentration and adenylate cyclase activity were it true that receptors and cyclase are permanently (pre)coupled in the manner depicted in Fig. 1 (and it is important to reemphasize here that they are not).

This equation relating k_{on}, k_i and EC_{50} would bear no relationship to an actual k_{on} if any one receptor could access an unlimited number of cyclases during one cycle of binding and unbinding (e.g. mobility of receptors was infinitely large). However, we have shown previously [15] that this is not the case: not only is mobility a limiting factor in the epinephrine concentration response, but also during the course of one cycle of epinephrine binding, a receptor is probably involved with at most one cyclase; during the time scale of one lifetime of a receptor–epinephrine complex, the receptor is either engaged with G protein in a way that is effectively private and non-promiscuous, or it is not engaged with G protein at all. The value for k_{on} derived above is thus related to a *minimum* value for k_{on}. Since we have made the assumption that every binding is an effective activation of a cyclase, i.e. that a receptor is always in the position of activating cyclase upon binding, then the actual value for k_{on} must be greater. If a receptor is in such a position only a fraction, ω, of the time [i.e. where ω is the fraction of the time ($\omega < 1$) when the receptor is engaged with G protein, by virtue of proximity, collision or encounter, and capable of activating G protein only if bound], then

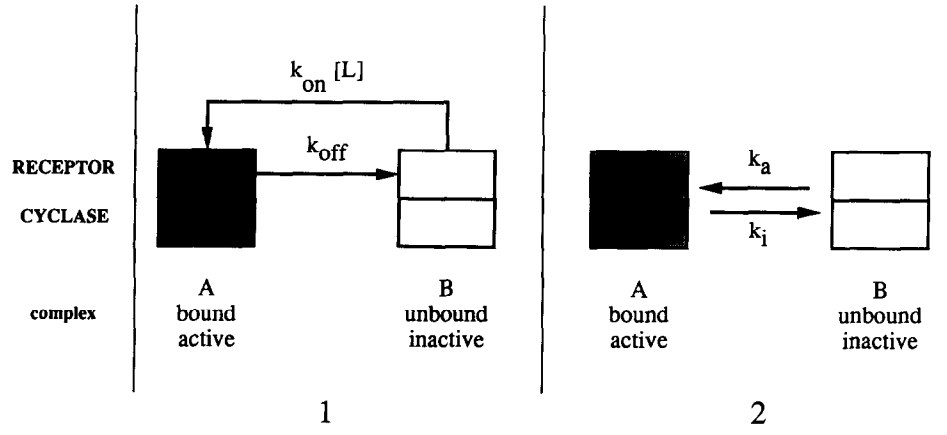

Fig. 1. Two-state model for G protein/adenylate cyclase activation assuming a precoupled receptor–cyclase complex and perfect efficiency for activation upon agonist binding. The process of agonist association (in 1) is equivalent to cyclase activation (in 2); the process of agonist dissociation (in 1) is equivalent to cyclase inactivation (in 2).

$$k_a = \omega k_{on}[L]$$

and thus

$$k_{on} = \frac{k_i}{EC_{50}\,\omega} > \frac{k_i}{EC_{50}}.$$

The value for k_i has been measured in our laboratory ($k_i = 1.4\,min^{-1}$) by a modification of the method described by Cassel *et ql.* [9]. Using epinephrine, accumulation of cAMP was measured at 5-sec intervals after addition of 10 nM epinephrine (=EC_{50}). The time course for cAMP accumulation is concave upwards at short times reflecting the delay in the attainment of the steady-state activity level for the adenylate cyclase. Thereafter a steady state is achieved in which the accumulation of cAMP is linear for about 1 min. Under these circumstances, extrapolation of the straight line portion of the accumulation curve back to the abscissa gives an intercept at $t = 1/(k_a + k_i)$. Given that the agonist concentration is equal to the EC_{50}, then $k_a = k_i$, and k_i is calculated directly from this intercept. The value for k_i is apparently (as expected) independent of the agonist used for its determination, as it has been obtained using a number of different agonists at their EC_{50} values. Using this value for k_i and given an EC_{50} of 10 nM:

$$k_{on} > 1.4\,min^{-1}/10^{-8}\,M = 1.4 \times 10^8\,M^{-1}\,min^{-1}.$$

Correspondingly, for $K_d = 2\,\mu M$, k_{off} is given by

$$k_{off} > K_d \cdot k_{on} = 280\,min^{-1}.$$

Neither the dissociation constant K_d nor the finite lifetime of the receptor–epinephrine complex is a factor for the calculation of k_{on} in the completely binding frequency-dependent model of activation given above. A variation on this model that takes these factors into consideration is presented below. A second variation of the model, in which activation is not instantaneous upon binding of ligand, is also presented. In both variations the rate constants for binding and dissociation are shown to correspond to those derived above using the simplest model.

Model including receptor–epinephrine complex lifetime

Here we consider a model in which the lifetime of the receptor–epinephrine complex is taken into account. The model (Fig. 2) is revised from the frequency-dependent model above. In this model it is assumed that: (1) receptor is continuously coupled to enzyme (G protein/adenylate cyclase); (2) the enzyme becomes active when the receptor becomes bound; and (3) the enzyme cannot become inactive while the receptor is still bound. Again in this model a cyclase molecule is activated every time a receptor becomes bound, i.e. the receptor is infinitely efficient at activation upon becoming bound. These assumptions lead to a formulation for the value of k_{on} in terms of the EC_{50}, K_d and k_i. As above, by taking into account the fact that a receptor is not continuously coupled to enzyme, it will be shown that the rate constants derived from this model are minimum values. The formulation is derived as follows:

In the model there are three states of the receptor–cyclase complex: bound (therefore) active (A); unbound active (B) and unbound inactive (C). The steady-state fraction of the complexes which are in each state is readily visualized on plots of cyclase activation and receptor binding (Fig. 3). With these assumptions the value for k_{on} can be solved by a mass balance of the three species A, B and C. At a given concentration of agonist L, the probability, P, that a given complex is in state (A) (bound and therefore also active) at any instant is given by

$$P(A) = \frac{[L]}{[L] + K_d} = \text{fraction bound} = \theta.$$

The fraction which is unbound but active [or the probability, P, that a complex is in state (B)] is the difference between the fraction f in the activated

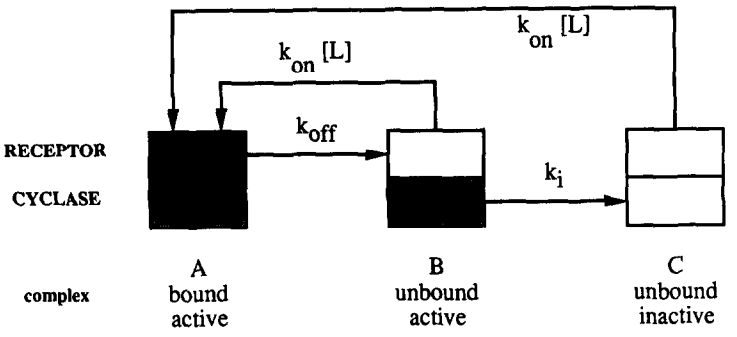

Fig. 2. Three-state model for adenylate cyclase activation assuming a precoupled receptor–cyclase complex. In this model the cyclase is active when the receptor is bound (state A) and has a fixed probability per unit time of remaining active after the receptor becomes unbound (state B). Some fraction of the complexes are both unbound and inactive (state C).

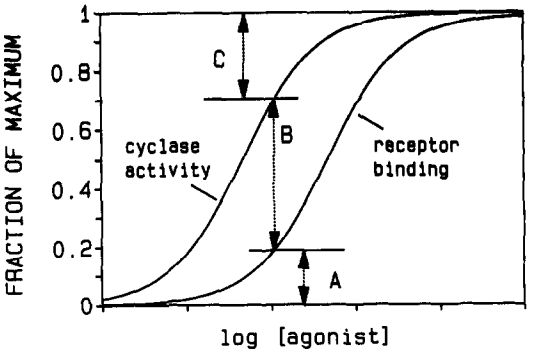

Fig. 3. Agonist concentration versus response (adenylate cyclase activity = rate of cAMP formation, as a fraction of maximum) and versus agonist binding (receptor occupancy as a fraction of maximum) for a theoretical agonist in the three-state model of Fig. 2. The relative lengths of the vertical lines A, B and C (· · · ·) represent the relative probabilities P for states A, B and C described in Fig. 2.

state and the fraction in state (A). Given the empirical relation

$$\text{fraction activated} = \frac{[L]}{[L] + EC_{50}} = f$$

then

$$P(B) = f - P(A).$$

The remainder of the complexes are in the inactive form:

$$P(C) = 1 - f = 1 - [P(A) + P(B)].$$

At steady state the rates of change of each species = 0:

$$0 = \frac{dA}{dt} = k_{on}[L](B + C) - k_{off}A$$

$$0 = \frac{dB}{dt} = k_{off}A - (k_{on}[L] - k_i)B$$

$$0 = \frac{dC}{dt} = k_i B - k_{on}[L]C.$$

Thus (from $dC/dt = 0$):

$$k_{on} = \frac{k_i}{[L]}\frac{P(B)}{P(C)} = \frac{k_i}{[L]}\frac{(1-\theta)}{(1-f)}.$$

Given this model, a value for k_{on} can be calculated as a function of [L] given k_i, since EC_{50} and K_d are known (i.e. θ and f are known for a given [L]). The constant k_{on} is, of course, not a function of [L], however; the computation is valid as [L]→ zero, where the cyclase activation rate becomes proportional to the rate of receptor binding (viz. where the probability of binding to a complex which is already active is zero). As [L]→zero, the formulation reduces to:

$$k_{on} = k_i\left(\frac{1}{EC_{50}} - \frac{1}{K_d}\right).$$

The results of this calculation for epinephrine (EC_{50} = 10 nM, $K_d = 2\,\mu M$) gives the estimate:

$$k_{on} > 1.4 \times 10^8/M/min.$$

Note that because of the difference in EC_{50} and K_d, the K_d (the factor introduced to account for receptor complex lifetime) contributes only insignificantly to the k_{on} value compared to the k_{on} calculated using the simpler binding frequency-dependent model. By the same arguments as those given above, this value for k_{on} is a minimum value; since a receptor is not at all times in a position to activate cyclase (i.e. *not* precoupled), then k_{on} would need to be greater in order to account for the observed concentration–response relationship.

Model including an additional rate-limiting step in cyclase activation

In the system described above, it is assumed that activation of cyclase is instantaneous upon binding of epinephrine to a receptor coupled to G protein/cyclase. Here we consider a model in which that assumption is relaxed, in order to demonstrate that the inclusion of an additional step for activation leads to a value for k_{on} which is at least as great as the minimum k_{on} values calculated using the prior models.

In this model (Fig. 4) the system is as before except for the addition of an activation step between binding and cyclase activation with the rate constant

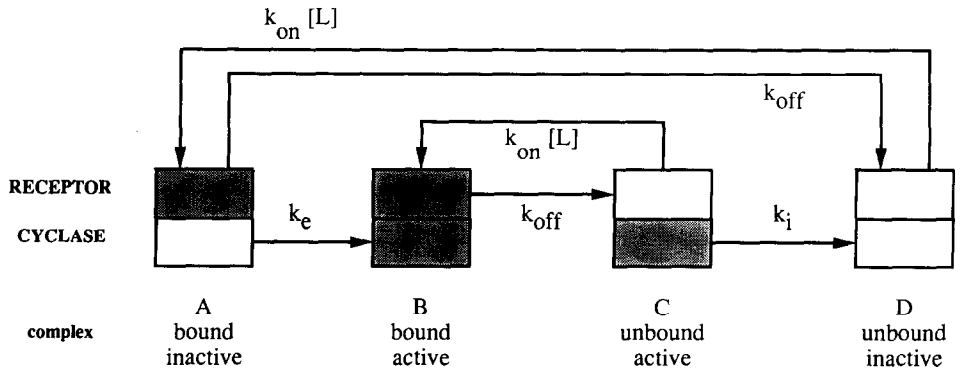

Fig. 4. Four-state model for adenylate cyclase activation assuming a precoupled receptor–cyclase complex. This model is an extension of the model shown in Fig. 2 to include an activation step with rate constant k_e.

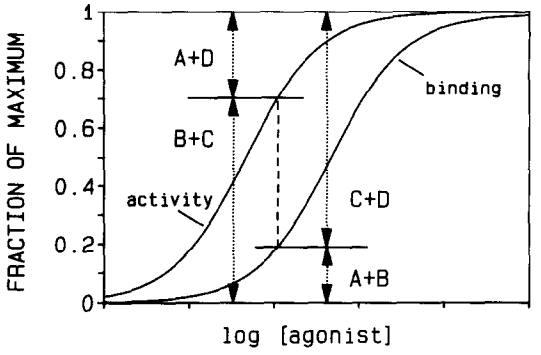

Fig. 5. Agonist concentration versus response (adenylate cyclase activity = rate of cAMP formation, as a fraction of maximum) and versus agonist binding (receptor occupancy as a fraction of maximum) for a theoretical agonist in the four-state model of Fig. 4. The relative lengths of the four vertical lines (· · · ·) represent the relative probabilities P for four combinations of states A, B, C and D described in Fig. 4.

k_e. Note that as $k_e \rightarrow \infty$, the system reduces to that shown in Fig. 2. The inclusion of an additional step in the model creates an additional state of the receptor–cyclase complex which is bound and inactive.

In the model there are four states of the receptor–cyclase complex: bound and inactive (A), bound and active (B), unbound and active (C) and unbound and inactive (D). At a given concentration of agonist L, the probability, P, of two combinations of states are given by the probabilities of being bound (receptor) or being active (cyclase):

$$\text{bound fraction} = P(\text{A or B}) = \frac{[\text{L}]}{[\text{L}] + K_d} = \theta$$

$$\text{active fraction} = P(\text{B or C}) = \frac{[\text{L}]}{[\text{L}] + \text{EC}_{50}} = f.$$

The fraction of the complexes which are in each state can be visualized on plots of cyclase activation and the receptor binding (Fig. 5). With these assumptions the value for k_{on} can again be solved by a mass balance of the four species A, B, C and D. At steady-state:

$$0 = \frac{\text{dA}}{\text{d}t} = k_{on}[\text{L}]\,\text{D} - (k_e + k_{off})\text{A}$$

$$0 = \frac{\text{dB}}{\text{d}t} = k_{on}\,\text{A} + k_{on}[\text{L}]\,\text{C} - k_{off}\,\text{B}$$

$$0 = \frac{\text{dC}}{\text{d}t} = k_{off}\,\text{B} - k_{on}[\text{L}]\,\text{C} - k_i\,\text{C}$$

$$0 = \frac{\text{dD}}{\text{d}t} = k_i\,\text{C} - k_{on}[\text{L}]\,\text{D} + k_{off}\text{A}.$$

These equations may be rearranged to give the formulation

$$\frac{\left(\frac{k_i}{k_e}k_{off} + k_{on}[\text{L}] + k_i\right)}{(k_{on}[\text{L}] + k_{off} + k_i)} = \frac{[\text{L}] + \text{EC}_{50}}{[\text{L}] + K_d}.$$

As $[\text{L}] \rightarrow 0$:

$$\frac{k_i\,(k_{off} + k_e)}{k_e\,(k_{off} + k_i)} = \frac{\text{EC}_{50}}{K_d}$$

or

$$k_{off} = k_i \frac{1 - \frac{\text{EC}_{50}}{K_d}}{\frac{\text{EC}_{50}}{K_d} - \frac{k_i}{k_e}}.$$

From this equation it can be seen that any finite value of k_e (i.e. existence of step with $k_e < \infty$) leads to an increase in k_{off}. Since $k_{on} = k_{off}/K_d$, this leads to a proportional increase in k_{on}. Thus, the value for k_{on} calculated using the simpler model is a minimum value for k_{on}. Note also that as $k_e \rightarrow \infty$:

$$k_{off} = k_i\left(\frac{K_d}{\text{EC}_{50}} - 1\right)$$

which is equivalent to

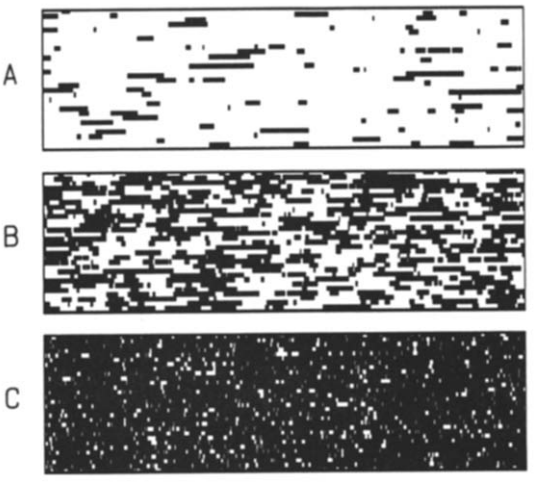

Fig. 6. Simulations of individual receptor binding at steady state for three concentrations of epinephrine (A, 0.1 K_d; B, K_d; C, 10 K_d). Each receptor is represented as a horizontal track in the figure (30 receptors in each case), moving through time (0.1 min from left to right); dark indicates bound, light indicates unbound (unoccupied). The initial condition for each track is determined by the steady-state probability (θ) of being bound or unbound (θ = [epinephrine]/[epinephrine) + K_d).

$$k_{on} = k_i \left(\frac{1}{EC_{50}} - \frac{1}{K_d} \right)$$

which result is the same as that derived from the simpler model.

Simulation of individual receptor binding using the rate constants

Given the kinetic rate constants k_{on} and k_{off}, the interaction of individual receptors with epinephrine over time can be examined by simulation. Given an initial state of being either bound or unbound, a change in state during the time interval Δt can be predicted from the kinetic equations:

Probability of becoming bound if unbound = k_{on} [epi] Δt

Probability of becoming unbound if bound = k_{off} Δt.

A simulation using these equations gives an on–off "history" of individual receptors (Fig. 6). It is apparent from the simulation that even at low occupancy the process of epinephrine binding and unbinding involves virtually all of the receptors within a relatively short time. For this reason, it has been pointed out that the label "spare" receptors for such a system (one in which a large response is obtained at low occupancy) can truly be a misnomer [17].

DISCUSSION

The analysis presented above for determining a minimum value for the association and dissociation rate constants for epinephrine is based on the empirical relationship between the binding of epinephrine to receptors and the adenylate cyclase-activating response elicited by bound receptors. In the S49 cell, a near-maximal response is obtained when receptors are occupied by epinephrine only a small fraction of the time ($EC_{50} \ll K_d$). Previous results suggested that a model equivalent to a transiently private receptor model would enable the rate constant for the association of epinephrine with β-adrenergic receptors to be estimated. Essentially we have shown previously [15] that at low epinephrine concentrations and low receptor occupancy, the diffusional rate of receptor–epinephrine complexes is insufficient to account for the measured rate of G protein/adenylate cyclase activation. Consequently, in the limiting case of vanishingly small concentrations of epinephrine, the rate of activation of adenylate cyclase cannot exceed the rate at which epinephrine binds to receptors. This being the case it is possible to set limits on the rates of epinephrine association and dissociation calculated from the adenylate cyclase activation using the transiently private receptor model. The model is formulated from the basic tenets of collision coupling, with the added assumption that it is unlikely that any one receptor can activate more than one cyclase during one binding cycle. The utility of the model relies ultimately on a short half-life ($t_{1/2}$) for the epinephrine–receptor complex, since at low concentrations of epinephrine the collision coupling model and the private receptor model become identical as the half-life of the bound receptor complex goes to zero (i.e. the maximum effective receptor/G protein collision rate, and hence cyclase activation rate, is identical to the binding rate when the bindng is of zero duration).

Previously, when questions of association and dissociation rates of epinephrine have arisen, it has been supposed simply that such rates were "fast", especially in cases where the nature of the experiments was such that half-lives of the receptor–agonist complex could be several seconds without affecting the interpretation of the data (e.g. in agonist/antagonist competition experiments [2]). In this study we have been able to set $t_{1/2}$ to an even shorter period. Given the value $k_{off} = 280$/min (using $K_d = 2\ \mu$M), the receptor–epinephrine complex $t_{1/2} = 0.15$ sec with a corresponding mean lifetime of the bound state = 0.22 sec. The effect of such a short half-life is that any degree of receptor occupancy is shared equally among the entire available receptor population within a relatively short period (on the order of seconds). For instance, at an epinephrine concentration of 2×10^{-7} M, for which overall occupancy is approximately 0.1, the half-time for the unbound state is less than 1.5 sec.

The model for the calculation of the rate constants k_{on} and k_{off} yields a formulation in which both constants are proportional to the inactivation rate constant k_i for adenylate cyclase, and inversely proportional to the value for EC_{50}. Thus, the precision with which k_i and EC_{50} are known directly affects the precision of the calculated values for k_{on} and k_{off}. We have used the value $k_i = 1.4$/min measured in S49 cells in our laboratory. There can be, however, some variation in this number in independent

experiments separated over periods of weeks (from 1/min to 3/min). Drifts on the order of a factor of two are also observed in the EC_{50} and K_d values (although the calculated value for k_{on} is not affected significantly by small differences in K_d because of the large separation between EC_{50} and K_d). Thus, on the basis of the precision and variability of the values for k_i and EC_{50}, we would place the value of k_{on} at a round figure of $k_{on} > 10^8$/M/min. This value for k_{on} is more than one order of magnitude less than the lower limit for a diffusion limited process for small substrates ($k > 10^9$/M/sec) [18, 19]. The value for the minimum k_{on} is also more than one order of magnitude more accurate than that made on the basis of the (arbitrarily defined) "rapidity" of binding given above. The corresponding value for k_{off}, given $K_d = 2\ \mu$M, is well above the maximum value (0.5/min) which is directly measurable experimentally [3].

The calculated value for $k_{on} = 10^8$/M/min can be compared to values determined for other β-adrenergic ligands. Meuller *et al.* [5] report the value for the rate constant $k_{on} = 10^8$/M/min for the interaction of isoproterenol with β-adrenergic receptors of polymorphonuclear leukocytes. Their approach was one in which the value for k_{on} was deduced from the functional effect of β-adrenergic agonists to inhibit the superoxide anion production response to chemotactic *N*-formyl peptide stimulation. This value for k_{on} which they calculated was reasoned to be a lower-limit (minimum) value, since it was assumed that the binding could not have occurred more slowly than the measured functional effects of binding. It should be noted that the K_d for isoproterenol binding in their study (40 nM) is remarkably different from the K_d for epinephrine binding in S49 cells (2 μM). Because of this difference the dissociation rate constant k_{off} for isoproterenol is much smaller (4/min); in contrast to epinephrine binding to S49 cells, for which the approach to steady-state binding (using Equation 1) takes place on a 0- to 3-sec time scale, the binding of isoproterenol to leukocyte receptors takes place on a much longer 0- to 60-sec time scale.

The calculated k_{on} value is also comparable to the association rate constant measured for the antagonist propranolol. We have measured previously $k_{on} = 4.0 \times 10^8$/M/min for propranolol binding to S49 cells ($K_d = 650$ pM) (unpublished data). In contrast to the results obtained by Contreras *et al.* [3], here the differences in the association rate constant k_{on} between agonist and antagonist appear to be slight, and the difference in affinities for these ligands appears to be attributable to differences in the dissociation rate constant k_{off}. Contreras *et al.* [3] calculated kinetic binding constants of β-adrenergic agonists and antagonists using the method of Motulsky and Mahan [4] to analyze changes in the time course of binding of a labeled ligand in the presence of a competing unlabeled ligand. Their experiments were done using L6 myoblast membranes in appropriately high concentrations of GTP. At 10°, k_{on} for isoproterenol ($K_d = 74$ nM) was calculated to be 10^6/M/min, which differed significantly from $k_{on} = 3 \times 10^8$/M/min for propranolol ($K_d = 110$ pM); at 25°, however, isoproterenol binding was found to be too rapid to be analyzed by this means. For comparison, the analysis presented here might be applied to a calculation of a minimum k_{on} for isoproterenol in the intact S49 cell ($EC_{50} = 2$ nM, $K_d = 200$–300 nM), although because of its higher affinity compared to epinephrine, the assumption that one binding cycle has at most one encounter between receptor and G protein may be a less valid one. With that caveat, the equations derived above give $k_{on} > 7 \times 10^8$/M/min for isoproterenol, similar to that for propranolol. However, the EC_{50} for isoproterenol in membrane preparations of S49 cells at 30° is 55 nM ($K_d = 660$ nM) which when applied in our equation gives $k_{on} > 2 \times 10^7$/M/min. This discrepancy from the value obtained by Contreras *et al.* is reasonable given the difference in preparations and temperatures.

The value for k_{on} for epinephrine in the intact S49 cell happens also to be similar to the value of k_{on} recently determined for the binding of epidermal growth factor (EGF) to the EGF receptor ($k_{on} = 1.8 \times 10^8$/M/min; $K_d = 600$ pM) [20].

The fact that the estimated values for the rate constants are comparable to those obtained in other systems supports the concept of the transiently private receptor. On the one hand, we have shown that a model which can explain the data on the relationship between binding frequency and adenylate cyclase response can be used to estimate the binding rate constants, taking the model as a given and true premise. On the other hand, one can say that the rate constants estimated using the model are a means of assessing the plausibility of the model, taking an estimated limit on the rate constants as a given and true premise. From this standpoint the model is supported by the fact that the rate constants are not different from "expected" values.

Two additional aspects of the system model should be noted. First, although it may appear that we have assumed a 1:1 ratio for G protein:cyclase (G:C), the precise assumption is rather that G activity and C activity are kinetically equivalent, as has been shown for turkey erythrocytes by Tolkovsky *et al.* [16]; that is, we have assumed that there is no C that is active independent of an associated active G. This is not equivalent to an assumption of a 1:1 ratio of the two species, and to our knowledge the actual ratio has not been measured accurately. Given the assumption of kinetic equivalence, there would be nonetheless no effect on the model calculations were this ratio different from 1:1 (and there is no reason to anticipate that it should be 1:1). Second, although we have attributed the rate constant for cyclase inactivation to the slow GTPase activity of G protein, this is essentially a useful description rather than an assumption of the model used for the calculation of the rate constants for binding. In the model we have assumed only that the kinetics for the process of inactivation can be modeled adequately by a first-order rate constant, even if that process is not solely and specifically equivalent to the process of GTP hydrolysis.

The rate constants for epinephrine binding have not been measured and are not directly measurable by conventional techniques. Previous to this analysis, the only idea one could gain of these rate constants

would be to use the "rule" that similar ligands of one receptor have similar association constants. One can then use the measured dissociation rates for slowly dissociating ligands to estimate those for other ligands by comparing their thermodynamic dissociation constants (K_d values). The present estimate for the minimum rate constants for epinephrine binding is based on measurements of a phenomenon (adenylate cyclase activation) which is quantitatively dependent on those constants. The half-time for adenylate cyclase activation (related to k_i) is many seconds and is relatively easy to measure. In the approach presented here this easily measurable time is related to the much faster and not directly measurable rate of dissociation of epinephrine from the receptor.

Acknowledgement—This work was supported by NIH NS-21338.

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