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The Kinetics of Competitive Radioligand Binding Predicted by the Law of Mass Action

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SUMMARY

Although equilibrium competitive radioligand binding studies are often used to characterize hormone and neurotransmitter receptors, the kinetics of such experiments have not been extensively explored. The interactions of the radioligand and competitor with the receptors can be described by two differential equations which can be solved to yield a single equation describing the binding of the radioligand as a function of time. This equation has several applications: First, it can be used to simulate competitive binding reactions under defined conditions. Second, fitting experimental data to this equation allows one to determine the association and dissociation rate constants of the competing ligand, parameters that cannot be derived from equlibrium experiments. Furthermore, this method can be used to determine the K_I of the competing drug from data acquired before equilibrium is reached. Third, mathematical analysis of the binding equation allowed us to answer two specific questions regarding the kinetics of competitive radioligand binding: how long such an incubation takes to equilibrate, and how the IC_{50} varies over time. The answers to these questions depended, to a large extent, on the relative values of the dissociation rate constants of the radioligand and competitor, which can be determined as noted above. When the competitor dissociates from the receptors more rapidly than the radioligand, the IC_{50} first decreases and then increases, but never has a value less than the K_I. At low radioligand concentrations, equilibrium is reached in the same amount of time required of the radioligand to dissociate completely from the receptors as determined in an "off-rate experiment." At higher concentrations of radioligand this time is halved. When the competitor dissociates from the receptor more slowly than does the radioligand, then the time required to equilibrate depends only on the dissociation rate constant of the competitor, and the IC_{50} decreases over time.

INTRODUCTION

Competitive binding experiments, in which a radiolabeled ligand competes with an unlabeled drug for binding to a receptor site, are widely used to characterize hormone and neurotransmitter receptors. Usually the incubation is allowed to reach equilibrium[3] before the experiment is terminated and the radioligand binding is determined. The properties of these equilibrium competitive binding experiments are well described, as are various methods for their analysis (1). In some experimental situations it is necessary or useful to examine competitive binding experiments before equilibrium is reached, but, although the kinetics of competitive binding have been partially described (2–4), several questions remain. Using a mathematial expression describing the kinetics of radioligand binding in the presence of a competing ligand, we addressed the following theoretical questions: How long does a competitive binding experiment take to reach equilibrium? How does a competitive binding curve change over time? How can the dissociation constant of a receptor for an unlabeled ligand be determined from non-equilibrium competitive binding studies?

THE MODEL

In this paper we consider only a very simple and widely used model in which the radioligand and competing drugs each bind reversibly to the receptors with specified kinetic constants and according to the law of mass action. This model can be expressed by the two binding reactions

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[3] Strictly speaking, equilibrium in never "reached"; rather it is asymptotically approached. In a practical sense, however, equilibrium is reached once the binding deviates from its ultimate equilibrium value by an unmeasurable and trivial amount. After five half-lives, this deviation is 3% of the equilibrium value.

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using the following symbols[4]: R = receptor, L = radioligand, I = competing drug (or inhibitor); RL = receptor-radioligand complex, RI = receptor-competing drug complex:

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL$$

$$R + I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} RI$$

Here k_1 and k_3 are the forward-, association-, or on-rate constants for the respective binding of radioligand and competitor to the receptors (in units of min^{-1} M^{-1}), and k_2 and k_4 are the respective reverse-, dissociation-, or off-rate constants (in units of min^{-1}). The equilibrium dissociation constant of the binding of radioligand to receptor (K_D) is defined to be k_2/k_1 (units of molar); the equilibrium dissociation constant of competitor to receptor (K_I) is likewise defined as k_4/k_3.

To simplify the equations, we have limited our model to the situation in which only a small fraction (<10%) of the radioligand and competitor bind to receptors. Thus throughout the experiment the concentrations of free (unbound) radioligand and competitor are constants approximately equal to their respective total concentrations. This situation is often referred to as "zone A" (1).

The binding reactions and the conservation of mass lead to the following equations:

$$d[RL]/dt = [L][R]k_1 - [RL]k_2$$

$$d[RI]/dt = [I][R]k_3 - [RI]k_4$$

$$[R] = N - [RL] - [RI]$$

(here $[R]$ is the concentration of free receptors, N is the total concentration of receptors)

The three equations above completely describe the kinetics of a competitive binding incubation. Solving these differential equations (Appendix 1) yields an expression defining the amount of radioligand bound to receptors ($[RL]$) as a function of time:

$$[RL] = \frac{Nk_1[L]}{K_F - K_S}\left[\frac{k_4(K_F - K_S)}{K_F K_S} + \frac{(k_4 - K_F)}{K_F}\exp(-K_F t) - \frac{(k_4 - K_S)}{K_S}\exp(-K_S t)\right] \quad (1)$$

Here the following new variables are used:

$$K_A = k_1[L] + k_2$$

$$K_B = k_3[I] + k_4$$

$$K_F = 0.5[(K_A + K_B + \sqrt{(K_A - K_B)^2 + 4k_1k_3[L][I]})]$$

$$K_S = 0.5[(K_A + K_B - \sqrt{(K_A - K_B)^2 + 4k_1k_3[L][I]})]$$

[4] The abbreviations used are: R, receptor; L, radioligand; RL, receptor-radioligand complex; I, competitor; RI, receptor-competitor complex; k_1, association rate of radioligand; k_2, dissociation rate of radioligand; k_3, association rate of competitor; k_4, dissociation rate of competitor; N, total concentration of receptors; K_A, K_B, K_F, K_S, clusters of constants defined in text; t, time; IC_{50}, concentration of competitor required to compete for half of the radioligand binding, [^{125}I]ICYP, [^{125}I]iodocyanopindolol.

The general properties of Eq. 1 are as expected. At time = 0, the equation reduces to zero; there is no radioligand binding. As equilibrium is approached, the two exponential terms approach zero and may be ignored. The equation then reduces to:

$$[RL] = \frac{Nk_1k_4[L]}{K_F K_S} = \frac{N[L]}{K_D\left(1 + \frac{[I]}{K_I} + \frac{[L]}{K_D}\right)}$$

In order to compare the binding of radioligand in the presence of competitor with the binding of radioligand alone, we also need the equation describing the binding of radioligand alone to the receptors (1):

$$[RL] = \frac{k_1N[L]}{K_A}[1 - \exp(-K_A t)] \quad (2)$$

Using Eqs. 1 and 2, one can easily program a computer to simulate the competitive interactions of ligand, competitor, and receptors for any particular set of kinetic constants and ligand, competitor, and receptor concentrations. By mathematically manipulating those equations, one can also solve more general problems, as we do under Appendix and discuss below.

WHEN IS EQUILIBRIUM ESTABLISHED?

In the absence of inhibitor, the rate at which the radioligand binds to receptor is determined by the exponential term $\exp(-K_A t)$. The half-life for this binding is $0.69/K_A$. After five half-lives, $3.5/K_A$, equilibrium is virtually reached as binding deviates from its true equilibrium value by less than 3%. In the presence of competitor the situation is more complicated. Several authors have pointed out that it takes longer for equilibrium to be established when an inhibitor is present (2–4), but no general rule defining how long it takes has been published.

Competitive binding experiments are commonly performed with a single concentration of radioligand and a variety of concentrations of competitor in order to generate a competitive binding curve. The time required for the incubations to reach equilibrium depends, in part, on the concentration of competitor present. We first consider the approach to equilibrium when the competitor concentration is equal to its equilibrium IC_{50}. The time required for this to occur depends on the relative values of k_2 and k_4 (Appendix 2), and we consider the two extremes: first when $k_4 \ll k_2$ and then when $k_4 \gg k_2$.

When the dissociation rate of the unlabeled competitor is much slower than that of the radioligand ($k_4 \ll k_2$), equilibrium at the IC_{50} is reached at $1.75/k_4$. Note that in this case the concentration and kinetic constants of the radioligand do not matter. This relationship is only useful experimentally when k_4 is known or can be estimated (see below).

In many experimental protocols the radioligand dissociates from receptors more slowly than does the competitor ($k_2 \ll k_4$). As shown in Appendix 2, the length of time required to reach equilibrium at the IC_{50} depends on the radioligand concentration. When the radioligand concentration is low ($[L] \ll K_D$), the time required for the radioligand binding to approach equilibrium is the

same in the absence of competitor as in the presence of competitor, $3.5/k_2$. As the radioligand concentration is increased infinitely, the time required to reach equilibrium is only halved to $1.75/k_2$ (Fig. 1; Appendix 2). In the absence of competition, however, the rate of radioligand binding increases linearly with radioligand concentration. Thus the higher the radioligand concentration, the greater the disparity between the time required for equilibrium to be reached in the presence of the competitor and the time required in its absence.

The above analyses assumed that the competitor was present at its equilibrium IC_{50}. As seen in Fig. 2, the slope of the competitive binding curve decreases slightly over time; thus the periphery of the curve may not be as close to equilibrium as is the middle of the curve. The time required for the entire curve to reach equilibrium completely depends on the slower of the two dissociation rate constants k_2 and k_4. When the radioligand dissociates more slowly ($k_2 < k_4$), full equilibrium is reached at $3.5/k_2$; equilibrium is reached most slowly at high concentrations of competitor, where very little radioligand ever binds. Conversely, in situations where the competitor dissociates more slowly ($k_4 < k_2$), equilibrium is reached at $3.5/k_4$, and equilibrium is reached most slowly at very low concentrations of competitor.[5]

These mathematical relationships can readily be applied in an experimental context. The value of k_2 is routinely determined in "off-rate" experiments; radioligand is bound to tissue and the rate at which it dissociates is determined after diluting the incubation mixture or after adding an excess of an unlabeled receptor-specific drug. The time for essentially all (97%) of the radioligand to dissociate is $3.5/k_2$. After incubating that long, all competitive binding experiments in which $k_2 \leq k_4$ (regardless of radioligand concentration) will have reached equilibrium. When high concentrations of radioligand ($\geq 10\ K_D$) are used and $k_2 \ll k_4$, equilibrium at the IC_{50} will have been reached by half that much time.

For some radioligands there will be no problem following the guideline derived above. For other radioligands, however, it may not be feasible to allow an incubation to proceed that long. For example, the dissociation rate constant (k_2) of [^{125}I]ICYP from *beta*-adrenergic receptors on intact S49 lymphoma cells is 0.0045 min^{-1} (5). A competitive binding experiment, using a high radioligand concentration, would require nearly 400 min to reach equilibrium. In many contexts this would be impractical. As can be seen in Fig. 4, the IC_{50} can change very slowly during the final phases of the experiment, and an acceptable approximation of the equilibrium IC_{50} may be attained in less than half the time required for equilibrium to be established. This is best determined experimentally with each particular system.

Altering the receptor concentration does not affect the time required for competitive binding curves to reach equilibrium (as long as the system in in a zone A). Nor does altering the receptor concentration affect the time required for equilibrium to be reached when radioligand alone binds to receptors. This is because altering the receptor concentration does not change any of the time-dependent terms in Eqs. 1 and 2. If, for example, one doubles the number of receptors present, the number of receptors bound by radioligand or competitor each minute will be doubled. But, since there are twice as many receptors present, the time required to reach equilibrium is unchanged.

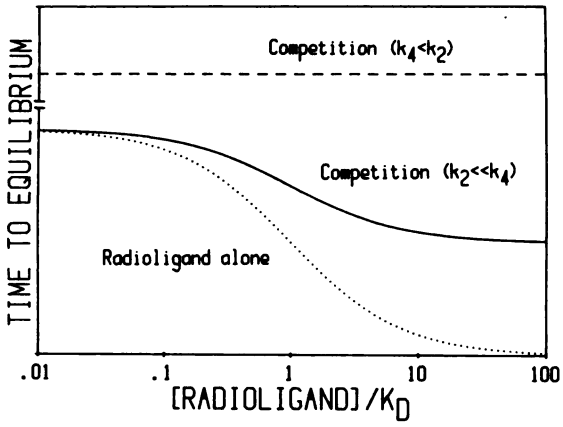

FIG. 1. *Effect of radioligand concentration on the time required for a competitive binding experiment to reach equilibrium*

The time required for radioligand binding to reach equilibrium in the presence of an IC_{50} concentration of competitor is plotted against radioligand concentration ([L]). When $k_4 \leq k_2$ this time does not depend on [L]; if $k_4 \ll k_2$ the equilibration time is $1.75/k_4$; if $k_4 = k_2$ the equilibration time is $3.5/k_4$. When $k_2 \ll k_4$ the situation is more complicated. At low radioligand concentrations the time required to reach equilibrium is $3.5/k_2$; this is the same as the time required for 97% of the radioligand to dissociate in an "off rate" experiment. When very large amounts of radioligand are used, the IC_{50} is much higher and the equilibration time is halved. Also shown is the time required for radioligand binding to reach equilibrium in the absence of competition.

COMPETITIVE BINDING CURVES BEFORE EQUILIBRIUM IS REACHED

Now that we have derived expressions defining the time required for competitive binding incubation to reach equilibrium, we turn to the next question: What do competitive binding curves look like before equilibrium is reached? Ehlert *et al.* (3) approached this question by performing numerical simulations of pre-equilibrium binding reactions. They concluded that the IC_{50} increases over time if $k_2 < k_4$ and decreases if $k_4 < k_2$. These generalizations were based on simulations of several binding curves at a few time points. We used an analytical approach to prove these generalizations. As shown below, the situation is more complicated at early time points.

To understand the changing positions of non-equilibrium competition curves, it is instructive to compare first the kinetics of radioligand binding in the absence and in the presence of competitor (Fig. 3A). Because competition binding curves are displayed as the ratio of radioligand binding in the presence of competitor to binding in its absence, we have plotted this ratio over time in Fig.

[5] Our definition of equilibrium, $3.5/k_2$ and $3.5/k_4$, may be practically irrelevant at extreme concentrations of competitor. At very high competitor concentrations, virtually no radioligand will *ever* bind, and "equilibrium" will be reached instantaneously. At very low concentrations of competitor, the competitor can be essentially ignored and the equilibration time is that of the radioligand alone, $3.5/K_A$. However, by deriving the equilibration time for these extreme cases, we assure that all parts of the competition curve will have equilibrated.

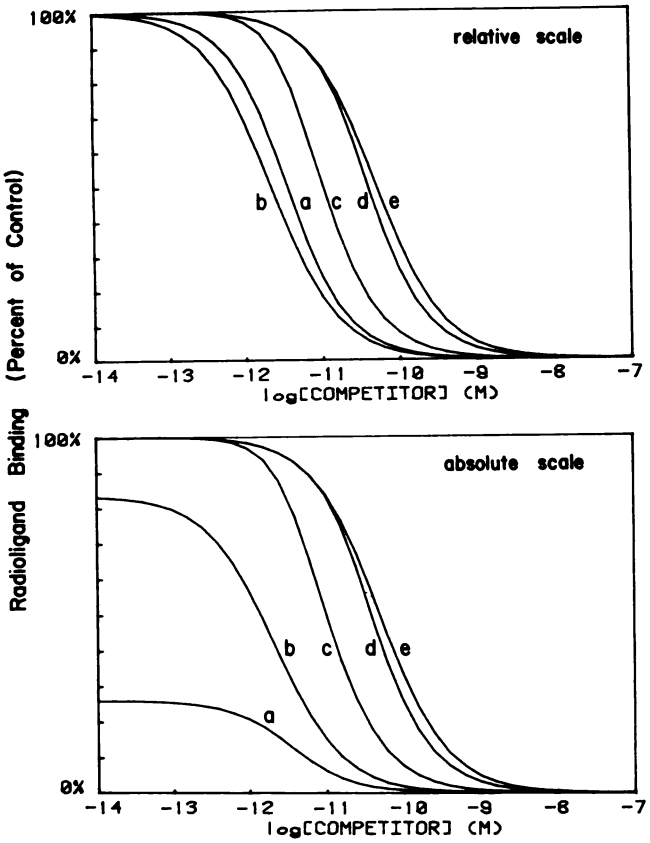

FIG. 2. *Change in apparent "slope factor" or "psuedo-Hill slope" over time*

Simulated competitive binding experiments are shown on a normalized scale (*top*) and on an absolute scale (*bottom*). To calculate these curves, the following values were used: [radioligand] = 35 pM, $k_1 = 5.86 \times 10^8$ min^{-1} M^{-1}, $k_2 = 0.0045$ min^{-1} [thus $K_D = 7.6$ pM; these constants are those of [^{125}I]ICYP binding to *beta*-adrenergic receptors on S49 cells (5)], $k_3 = 1000k_1$, $k_4 = 100k_2$. The curves were calculated at the following time points (minutes: $a = 1$, $b = 6$, $c = 36$, $d = 216$, and $e = 1296$. The respective "slope factors" (calculated at the IC_{50} of each curve) are 1.1, 1.0, 1.2, 1.2, and 1.0.

3B. At the very earliest time points the binding of radioligand is unaffected by the presence of (unbound) competitor; thus binding in the presence of competitor is 100% of the binding in its absence. This ratio immediately decreases and eventually reaches its equilibrium value.

When the radioligand dissociates from the receptors more rapidly than does the competitor ($k_2 > k_4$), the binding of radioligand in the presence of the competitor overshoots its equilbrium value: at some intermediate time points there is more radioligand bound to receptors than there will be at equilibrium (ref. 2; Appendix 3). Expressed as a percentage of radioligand binding in the absence of competitor, however, the radioligand binding in the presence of the competitor constantly decreases, as is shown in Fig. 3B.

When the radioligand dissociates more slowly than the competitor ($k_2 < k_4$), the specific binding does not overshoot its equilibrium value but rather monotonically approaches that equilibrium (Fig. 3A). Expressed as a percentage of the radioligand binding in the absence of competitor, however, the binding is biphasic. First the percentage drops, then it increases, as shown in Fig. 3B.

At equilibrium the properties of a competitive binding curve are determined by the K_I of the competitor, and every competitor with a given K_I will yield the same equilibrium competitive binding curve regardless of the individual values of k_3 and k_4 ($K_I = k_4/k_3$). Before equilibrium is reached, however, the kinetics of competitive binding depend on both k_3 and k_4. To demonstrate this point, we have plotted the IC_{50} versus time for several

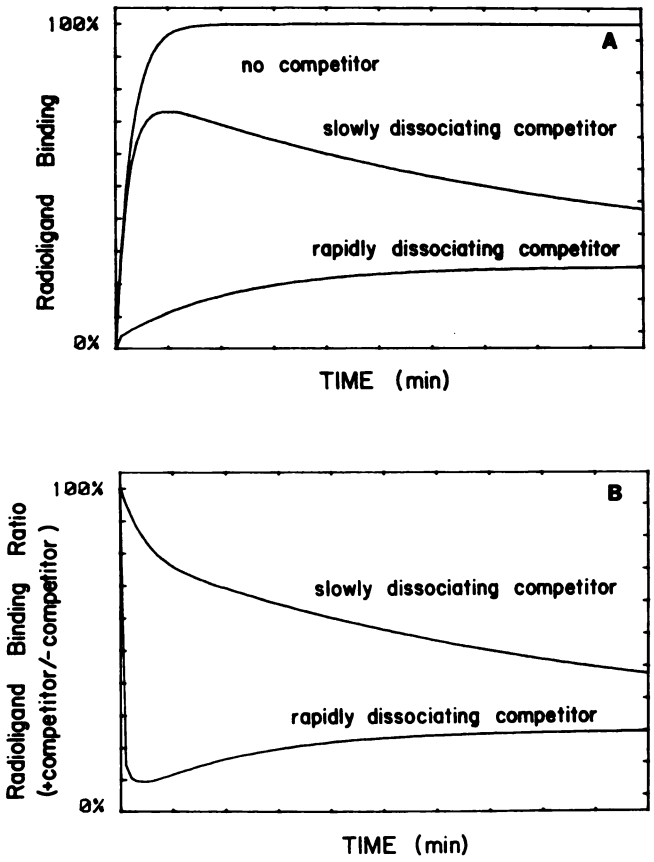

FIG. 3. *Binding of a radioligand in the absence and presence of a competitor*

A. The binding of a radioligand to receptor is shown in the absence of competitor (*top curve*), in the presence of a competitor that dissociates from the receptors more slowly than does the radioligand ($k_4 < k_2$; *middle curve*), and in the presence of a competitor that dissociates more rapidly than does the radioligand ($k_2 < k_4$; *bottom curve*). The vertical axis is radioligand binding relative to the equilibrium binding of radioligand alone. Note that equilibrium is reached more slowly in the presence of competitor. In the case of the slowly dissociating competitor, equilibrium has not yet been established at the right of the curve shown; at equilibrium this curve will merge with the curve of the rapidly dissociating competitor.

B. At each time point the amount of radioligand binding in the presence of rapidly dissociating (*upper curve*) or slowly dissociating (*lower curve*) competitior is displayed as a percentage of the binding of the radioligand alone at that time point. The following values were used to calculate these curves: $k_1 = 1.0 \times 10^8$ min^{-1} M^{-1}, $k_2 = 0.037$ min^{-1}, [radioligand] = 3 nM, [competitor] = 100 nM. [These values are those of [^{3}H]yohimbine binding to *alpha*$_2$-adrenergic receptors on platelets (8)]. For the rapidly dissociating competitor, $k_3 = k_1$ and $k_4 = 10k_2$. For the slowly dissociating competitor, $k_3 = k_1/100$ and $k_4 = k_2/10$. Thus the K_I was the same in each case (3.7 nM). The last time point shown is 100 min.

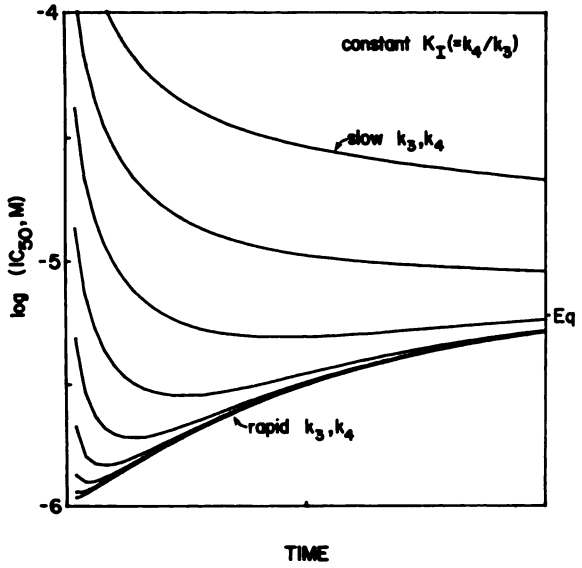

FIG. 4. *Change in the position of competitive radioligand binding curves over time*

The IC_{50} of a competitive binding curve is plotted against time. Maintaining a constant K_I, the association and dissociation rates of the competitor were varied to create the family of curves shown. In the *top curve*, the competitor associates and dissociates slowly; these rates are proportionately more rapid in the *lower curves*. To calculate the curves shown, the following values were used: $k_1 = 5.86 \times 10^8$ min^{-1} M^{-1}, $k_2 = 0.0045$ min^{-1}, and [radioligand] = 35 pM (same as Fig. 2). In the *top curve*, $k_3 = 10^3$ min^{-1} M^{-1}, $k_4 = 10^{-3}$ min^{-1}, and the K_I is therefore 10^{-6} M. Each succeeding curve was generated by increasing both k_3 and k_4 by half an order of magnitude. The bottom curve, therefore, has $k_3 = 10^7$ min^{-1} M^{-1} and $k_4 = 10$ min^{-1}. A computer calculated the entire competitive binding curve for each set of rate constants at each time point using Eq. 1, and found the IC_{50}. At equilibrium all of the curves converge with an IC_{50} of 5.6 μM. The last time point shown is 200 min.

The lowest curves shown ("rapid k_3, k_4") represent a common situation: the radioligand dissociates much more slowly than the competitor, but does not associate much faster. For these curves the IC_{50} is $\sim K_i$ at early time points and gradually increases to its equilibrium value defined by the Cheng and Prussoff equation (9), $IC_{50} = K_I(1 + [L]/K_D)$. Thus, when $[L] \ll K_D$, the IC_{50} will be nearly constant over time.

combinations of k_4 and k_3 yielding the same K_I (Fig. 4). In all of the curves the inhibition of radioligand binding at equilibrium is identical; only the kinetics of inhibition differ. As k_3 and k_4 increase, the initial decrease in the IC_{50} becomes more pronounced. In the most extreme case, when k_3 and k_4 are extremely fast, the minimum IC_{50} occurs instantaneously and has a value of K_I (Appendix 4). In all other cases, that minimum IC_{50} is larger and occurs later.

These findings are extended to entire competitive binding curves in Fig. 5. As Ehlert *et al.* (3) demonstrated, when $k_4 < k_2$, the IC_{50} of the competitive binding curve gradually decreases over time; the curve moves to the left. If, however, $k_4 > k_2$, then the IC_{50} will first decrease and later increase. That initial decrease in the IC_{50} may occur quickly and one may therefore observe only the later increase, as Ehlert *et al.* (3) did. In this case the minimum (leftmost) value of the IC_{50} will be the K_I. In all other cases the minimum IC_{50} will be greater than the K_I.

"Slope factors" or "pseudo-Hill slopes" are used to describe the shape of a competitive binding curve. We have simulated many pre-equilibrium competition curves on a computer and calculated the apparent slope factor

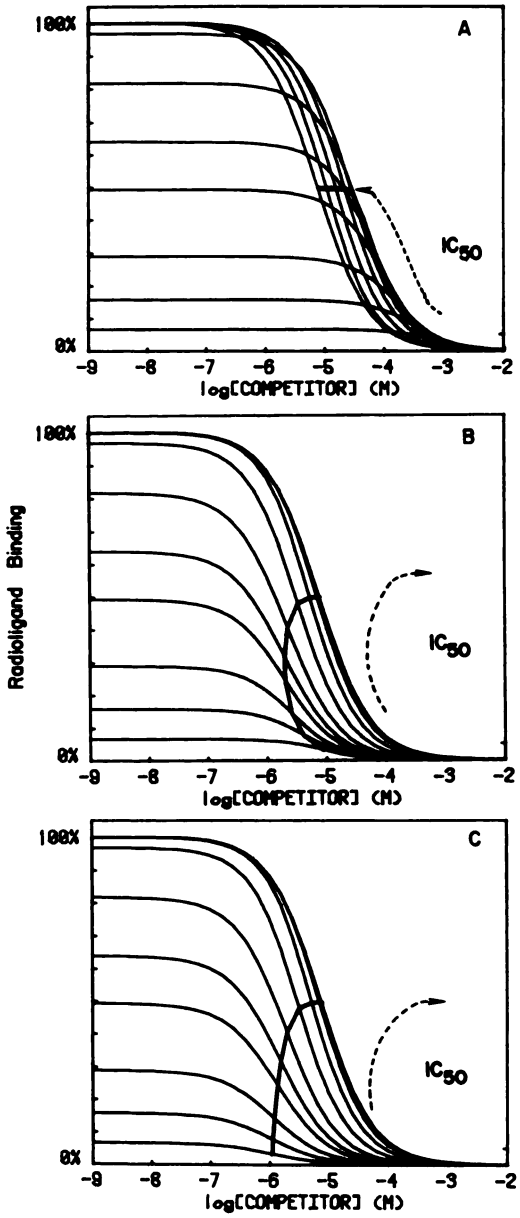

FIG. 5. *Change in competitive binding curves over time*

The constants used in Fig. 4 were used to calculate competitive binding curves at the following times (minutes: 2, 5, 10, 20, 30, 50, 100, 200, 400, 800, and 10,000.

A. The change in the competitive binding curve over time is shown for the case where $k_4 < k_2$. These curves were generated using the values noted above for the *topmost curve* of Fig. 4 ($k_4 = 10^{-3}$ min^{-1}). The *heavy line* connects the IC_{50} values; note that these values decrease over time.

B. Competitive binding curves were generated to match the middle curve of Fig. 4 ($k_4 = 10^{-1}$ min^{-1}). Note that the IC_{50} first decreases, then increases.

C. Here the competitive binding curves are plotted to match the *bottom curve* in Fig. 4 ($k_4 = 10$ min^{-1}). Here the initial decrease in IC_{50} occurs instantaneously and the IC_{50} increases over time. The initial and minimum IC_{50} is 10^{-6} M, which equals the K_I of the competitor.

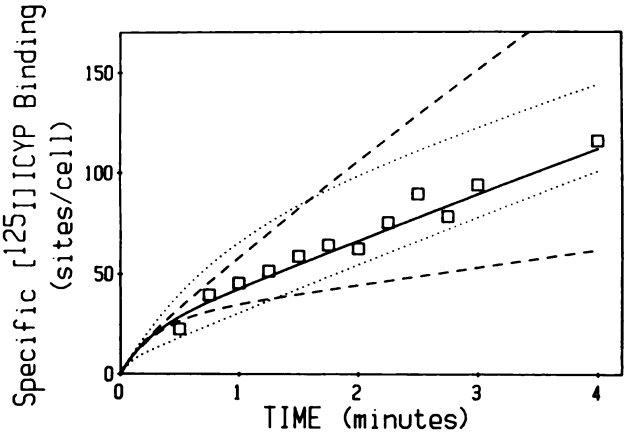

FIG. 6. *Determining the K_I from non-equilibrium competitive binding experiments*

To demonstrate the feasibility of determining the K_I of a competing ligand from non-equilibrium data, we analyzed the kinetics of [^{125}I] ICYP binding to *beta*-adrenergic receptors on intact S49 lymphoma cells in the presence of 1 nM propranolol. Using methods published elsewhere (5), ICYP and propranolol were added simultaneously to the cells and the specific ICYP binding was determined at various times between 0.5 and 4 min thereafter. The data were fit to Eq. 1 in the text using a Marquardt nonlinear least-squares regression program available from Tektronix (10). The program was given the following constants that were determined previously or set experimentally: $k_1 = 2.05 \times 10^9$ M^{-1} min^{-1} (determined in a parallel experiment), $k_2 = 0.0045$ min^{-1}, $[R] = 1200$ sites/cell (3.3 pM), $[I] = 1$ nM, and $[L] = 43$ pM. The computer determined the following values: $k_3 = 3.1 \pm 0.5 \times 10^9$ min^{-1} M^{-1}, and $k_4 = 1.01 \pm 0.26$ min^{-1}, yielding a $K_I(k_4/k_3)$ of 0.33 nM, and drew the *solid curve* shown.

To demonstrate the sensitivity of the technique, we also have shown how much the radioligand binding would differ if k_3 and k_4 were different. The *dotted lines* show the binding predicted assuming that the K_I was the same, but that the values of both k_3 and k_4 were varied either a half-order magnitude higher (*above*) or lower (*below*) than the values determined by the program. These curves are clearly resolved from the experimental points. The *dashed lines* show the binding predicted if k_4 alone were increased (*below*) or decreased (*above*) half an order of magnitude, thus altering the K_I.

for each. These simulations used a variety of kinetic constants, and the slopes (calculated at the IC_{50}) were always between 1.0 and 1.3. It is noteworthy that the pre-equilibrium slope factor was never less than 1 for curves that at equilibrium have a slope of 1.0. This can also be seen in Fig. 2.

DETERMINING THE K_I FROM KINETIC DATA

Equilibrium competitive binding curves are often used to determine the dissociation constant (K_I) of a receptor for an unlabeled ligand. As shown above, several hours may elapse before equilibrium is achieved in some receptor systems. During these hours other unavoidable events may occur that may affect the results. For example, the ligand or receptors may degrade, target cells may die, or the composition of the incubation mixture may change. In these situations it would be desirable to determine the K_I in a shorter period of time. Equation 1 makes this possible. The kinetics of radioligand binding in the presence of a competitor can be measured at many time points and the results are described by Eq. 1. In this equation the only unknowns are k_3 and k_4, as k_1 and k_2 are readily determined in standard experiments and the concentrations of radioligand, competitor, and receptor are set by the experimenter. Any general-purpose curve-fitting algorithm can therefore be used to fit Eq. 1 to the experimental data and determine k_3 and k_4, which together yield K_I (k_4/k_3).

In Fig. 6 we illustrate the feasibility of this approach for determining K_I. Here we have determined the K_I of *beta*-adrenergic receptors on S49 lymphoma cells for (−)-propranolol in a 4-min experiment. The result (0.3 nM) is similar to that determined in conventional equilibrium competitive binding experiments lasting 2 hr (0.2 nM; ref. 5). Moreover, the kinetic analysis yielded values for k_3 ($3.1 \pm 0.5 \times 10^9$ min^{-1} M^{-1}) and k_4 (1.0 ± 0.26 min^{-1}) that can not be determined from equilibrium experiments.

DISCUSSION

The equations and simulations were based on a simple molecular model incorporating the following assumptions: (a) a single class of noninteracting receptors is present that binds the radioligand and competitor reversibly; (b) these binding reactions follows the law of mass action; (c) radioligand and competitor cannot bind simultaneously to a single receptor binding site; (d) only a small fraction of the radioligand and competitor binds to receptors (zone A); (e) radioligand and competitor are simultaneously exposed to the receptors; and (f) the properties of all free receptors are identical whether or not they once bound ligand or competitor. This is a simple model, and a more complex model may be required in some experimental situations. Nevertheless, the model of simple competitive interactions incorporating these assumptions is commonly accepted as the basis of standard methods for analyzing competitive binding experiments.

Our analyses and discussion were based around radioligand binding experiments. The mathematics, however, are identical for any situation in which two ligands compete for binding to a single population of receptors, and the binding of one of those ligands is measured. Portions of our discussion may therefore apply to other situations such as radioimmunoassays, fluorescent binding assays, and competitive antagonism of pharmacological responses.

The theoretical analyses described in this paper apply in four experimental situations:

1. When establishing an experimental protocol for competitive radioligand binding experiments one must decide how long to allow the incubation to proceed in order to attain equilibrium. The analyses of this paper make it clear how to set the duration of the incubation. When the competitor dissociates from the receptor faster than does the radioligand, the time required to attain equilibrium is determined by the dissociation rate of the radioligand. Thus the time required for the radioligand to dissociate from receptors in an "off-rate" experiment is the same as the time required for a competitive experiment to reach equilibrium. Often investigators use an "on-rate" experiment to determine the time required. This will yield the correct result only if a very low concentration of radioligand is used (the observed "on

rate" is K_A [$= k_1[L] + k_2$], which approximates k_2 only when $[L] \ll K_D$). As noted above and in the legend to Fig. 4, an acceptable approximation of the IC_{50} can be attained in less than half the time required to reach equilibrium.

2. Several authors have demonstrated that it can take longer for radioligand binding to reach equilibrium in the presence of a competing drug than in its absence, and we have now quantitated the relationship. Thus, if an experimental protocol is based on the minimal time required for the binding of the radioligand alone to reach equilibrium, competitive binding experiments will be terminated before equilibrium is established. However, this fact is not well known, and some published competitive binding curves may have been obtained under non-equilibrium conditions. The relationship derived in this paper allow one to determine whether the apparent K_I values determined by these non-equilibrium curves are likely to be over- or underestimates of the true K_I.

3. Recent experiments by ourselves and others have demonstrated that *beta*-adrenergic agonists appear to bind transiently to *beta*-adrenergic receptors on intact cells with a high affinity, and that this binding "desensitizes" the receptors so as to decrease their later affinity for the agonists (5–7). This transient high-affinity binding is observed during the first few minutes of the competition between agonist and ligand, long before equilibrium is reached. At equilibrium the agonist appears to compete for radioligand binding with a low affinity and in a manner essentially consistent with the law of mass action. The anomolous behavior of agonist binding is observed only in kinetic experiments. A full theoretical analysis of this transient high-affinity binding has not yet been published. As a first step in analyzing such data, it is necessary to demonstrate that the data are not compatible with a simple model of competitive binding based on the law of mass action. The best way to demonstrate that the early competition data cannot be explained by the law of mass action is to compare directly the observed data with the theoretical predictions (5). In addition, the generalizations derived in this paper allow one to be certain immediately that an early competition curve is inconsistent with the law of mass action if the early IC_{50} is less than equilibrium K_I, or if the early slope factor is less than 1.0 (and the equilibrium slope factor is equal to 1).

4. It may not be feasible to allow an incubation to proceed long enough for equilibrium to be established if, for example, the ligand or receptor degrades, or the target cells die. Under such circumstances, the experimenter may be forced to terminate the binding incubations before equilibrium is established. We have shown how to determine the K_I using an experimental protocol that can be completed long before equilibrium is reached. Moreover, this technique uniquely allows one to determine the individual values of the association and dissociation rate constants of an unlabeled compound that determine the K_I.

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APPENDIX: MATHEMATICAL DETAILS

1. *Solution to the Differential Equations*

Defining y as $[RL]$ and x as $[RI]$, and setting both equal to zero initially, the differential equations were transformed by the method of Laplace:

$$s\hat{y} = Nk_1[L]/s - k_1[L]\hat{x} - k_1[L]\hat{y} - k_2\hat{y}$$

$$s\hat{x} = Nk_3[I]/s - k_3[I]\hat{x} - k_3[I]\hat{y} - k_4\hat{y}$$

Solving the second equation for $\hat{x}$ and inserting into the first yields (after some rearranging):

$$\hat{y} = \frac{NK_1[L]}{(s + K_F)(s + K_S)} + \frac{NK_1K_4[L]}{s(s + K_F)(s + K_S)}$$

Back-transforming yields Eq. 1 in the text. The intervening algebraic steps make use of the facts that $K_F + K_S = K_A + K_B$ and $K_FK_S = K_AK_B - k_1k_3[L][I]$. Arányi (2) has published a similar derivation.

2. *How Long Does It Take for a Competitive Binding Incubation to Reach Equilibrium at the IC_{50}?*

At the IC_{50}, $[I] = (k_4/k_3)(1 + [L]/K_D)$. In Eq. 1, $[I]$ and k_3 only appear as a product, the pseudo-first-order rate constant, $k_3[I]$. Thus, for a known K_D, a fixed $[L]$ and $[I] = IC_{50}$, $k_3[I]$ is a simple function of k_4. Similarly, for a radioligand of known K_D and fixed concentration $[L]$, the pseudo-first-order association rate constant, $k_1[L]$, is a simple function of k_2. Therefore the kinetics of binding may be described in terms of k_2 and k_4.

The time required for equilibrium to be achieved depends heavily on the relative values of k_2 and k_4. We consider first the case in which $k_2 \ll k_4$, then the case in which $k_2 \gg k_4$.

I. $k_2 \ll k_4$. The amount of time required for a competitive binding incubation to reach equilibrium depends, in part, on the radioligand concentration. We consider the two extremes, when the radioligand concentration is very low and when it is very high.

(a) Very low radioligand concentration: here $[L] \ll K_D$:

$$[I] = IC_{50} = K_I([L]/K_D + 1) \simeq K_I$$

Because $k_3[I]$ $(= k_4)$ is much larger than $k_1[L]$, the competitor will bind rapidly and the radioligand binding will take longer. Thus the competitor will always be nearly at equilibrium with free receptors, and we need only consider the binding of the radioligand:

$$d[RL]/dt = k_1[L][R] - k_2[RL]$$

$$[R] = (N - [RL])/2$$

(half of the receptors not occupied by radioligand will be bound to competitor because the competitor is present at its K_I and equilibrates rapidly).
Solving for $[RL]$:

$$[RL] = N/2(1 - \exp(-k_1[L] - k_2t))$$

$$\simeq N/2(1 - \exp(-k_2t))$$

The half-life is $0.69/k_2$; equilibrium is achieved at $3.5/k_2$. This is the same amount of time required for the

binding of radioligand alone, when it is present at very low concentration.

(b) Very high radioligand concentration: here $[L] \gg K_D$:

$$[I] = IC_{50} = K_I([L]/K_D + 1)$$

The pseudo-first-order on-rate of the radioligand, $k_1[L]$, can be expressed as $k_2[L]/K_D$). Similarly, the pseudo-first-order on-rate of the competitor, $k_3[I]$, can be expressed as $k_4([L]/K_D + 1)$. Given that $k_4 \gg k_2$, the competitor will therefore bind to the receptor much faster than will the radioligand. Thus again the competitor will reach equilibrium with the unoccupied receptors more rapidly than the radioligand will. Equilibrium will be established as the radioligand reaches equilibrium with the free receptors.

$$d[RL]/dt = k_1[L][R] - k_2[RL]$$
$$[R] = N - [RL] - [RI]$$

Because the competitor will always be virtually at equilibrium with the free receptors,

$$[RI] = [R][I]/K_I$$

From the definition of IC_{50},

$$[I]/K_I = [L]/K_D + 1 \simeq [L]/K_D$$

(because $[L] \gg K_D$). Substituting

$$[R] = [RI]K_I/I = [RI]K_D/[L],$$
$$[R] = (N - [RL])K_D/[L]$$
$$d[RL]/dt = k_2N - 2k_2[RL]$$

After integrating,

$$[RL] = N/2(1 - \exp(-2k_2t))$$

Therefore, the half-life is $0.35/k_2$ and equilibrium is reached at $1.75/k_2$ min. Thus, by increasing the radioligand concentration, the time required to reach equilibrium is halved. Why cannot the reaction be "pushed" faster? The rate at which the radioligand binds is proportional to both its concentration and the number of free receptors. When the radioligand concentration is increased, the concentration of competitor must also be increased (so that it remains as its IC_{50}), and the number of free receptors decreases. The product of radioligand concentration times free receptor concentration can at best be doubled by increasing the concentration of the radioligand.

II. $k_2 \gg k_4$. In this case the competitor will bind much more slowly than radioligand. We can therefore consider the free receptors and radioligand always to be at equilibrium. The time required for the entire competitive binding incubation to reach equilbrium is therefore the time required for the competitor to reach equilibrium with the free receptors. The math is similar to that above:

$$d[RI]/dt = k_3[I][R] - k_4[RI]$$
$$[R] = N - [RI] - [RL]$$
$$[RL] = [R][L]/K_D$$

substituting, $[R] = (N - [RI]/(1 + [L]/K_D)$ and $d[RI]/dt = k_4N - 2k_4[RI]$. Integrating, $[RI] = N/2(1 - \exp(-2k_4t))$.

Equilibrium is therefore reached in $3.5/2k_4 = 1.75/k_4$ min. Note that in this case the concentration of radioligand is irrelevant.

Determining the duration of time required to reach equilibrium depended largely on considering the relative rates at which radioligand and competitor bind to the receptors. Another approach is to analyze the time dependence of Eq. 1. The binding described by that equation will reach equilibrium as the slower exponential term involving K_S reaches equilibrium. Evaluating K_S numerically with various values for the kinetic constants and $[L]$ yielded conclusions identical with those derived above.

3. *Relationship of* k_2, k_4, k_F, K_S

When is $K_S > k_4$? Expanding K_S and rearranging yields

$$\sqrt{(K_A + K_B)^2 - 4K_AK_B + 4k_1[L]k_3[I]} < 2k_4 - (K_A + K_B)$$

Therefore, $-K_AK_B + k_1[L]k_3[I] > (k_4)^2 - k_4K_A - k_4K_B$.

Note that squaring the negative expressions caused the sign of the inequality to change. Simplifying this expression yields $k_4 < k_2$. Similarly $K_S > k_2$ when $k_4 > k_2$.

When is $K_F > k_4$ or $K_F > k_2$? Similar algebra leads to a tautology; therefore, K_F is always greater than k_2 and k_4.

Thus K_F is always greater than k_2 and k_4; K_S is always between k_2 and k_4.

4. *Proof That the Binding of Radioligand "Overshoots" Its Equilibrium Value if* $k_4 < k_2$

In Eq. 1 the $[RL]$ is defined by the sum of its equilibrium value plus two exponential terms. When these terms are positive, $[RL]$ will exceed that equilibrium value. This will occur when

$$\frac{k_4 - K_F}{K_F}\exp(-K_Ft) - \frac{k_4 - K_S}{K_S}\exp(-K_St) > 0$$

Because K_F is always greater than k_4, the first term will always be negative. The second term will make a positive contribution when $k_4 < K_S$; this occurs when $k_4 < k_2$. This is a sufficient condition for the entire sum to be positive at long time points, because $\exp(-K_Ft)$ will approach zero at these times.

5. *What Is the Minimum Ratio of Binding of Radioligand in the Presence of Competitor Compared with Binding in Its Absence?*

As shown in Fig. 3B, the binding ratio dips below its equilibrium value only if $k_2 < k_4$. This dip is most pronounced when k_3 and k_4 are large, as seen in Fig. 4. In the most extreme case, $K_B \gg K_A$ and $K_S = K_A$ and $K_F = K_B$. We cannot evaluate the binding ratio at time zero because there is no binding (division by zero), but we can evaluate the ratio at the earliest time dt. Because $[RL] = 0$ at time zero, $[RL]$ will equal the derivative of $[RL]$ with respect to time at the earliest time points.

Without competitor, $[RL]$ at early time points will be

$$[RL] = d[RL]/dt = Nk_1[L](1 - \exp(-K_A)) \simeq Nk_1[L]$$

In the presence of competitor, $[RL]$ will be

$$[RL] = d[RL]/dt = (NK[L]/(K_F - K_S))[(k_4 - K_S)\exp(-K_S t) - (k_4 - K_F)\exp(-K_F t)]$$

In the most extreme case, $K_F \gg K_S$ and at the earliest time point $\exp(-K_F) \simeq 0$ and $\exp(-K_S t) \simeq 1$. Therefore, $[RL] = NK_1[L](k_4 - K_S)/(K_F - K_S) \simeq Nk_1[L]k_4/K_F$. The ratio therefore is $\simeq k_4/K_F \simeq k_4/K_B \simeq K_I/([I] + K_I)$. This ratio will be 1:2 when $[I] = K_I$. By definition, when this ratio is 1:2, $[I] = IC_{50}$. In other words, at the earliest time points $IC_{50} = K_I$. This relationship was derived for the extreme case in which $K_B \gg K_A$. In less extreme cases, $IC_{50} > K_I$ initially. Under no circumstance can IC_{50} be less than the K_I.

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