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Estimation of the Affinities of Allosteric Ligands Using Radioligand Binding and Pharmacological Null Methods

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SUMMARY

The theoretical basis for using radioligand binding and pharmacological techniques to estimate the dissociation constants of drugs which interact allosterically with receptors is described. This theory predicts that an allosteric ligand changes the affinity of another ligand which binds at the primary recognition site on the receptor complex without affecting the binding capacity of the primary ligand. The magnitude of this effect depends on the amount of cooperativity (positive or negative) between the binding of ligands at the allosteric and primary recognition sites. It is possible to estimate the dissociation constant of an allosteric ligand by measuring its effect on the binding of a radioligand at a fixed concentration. In this situation, the dissociation constant of the allosteric ligand can be calculated from the concentration of ligand which causes half of its maximal effect on radioligand binding. The effects of an allosteric ligand on the pharmacological responses to an agonist can be attributed to changes in affinity and intrinsic efficacy of the agonist-receptor complex. If the agonist used in the pharmacological experiments has a large receptor reserve, or if the allosteric ligand only influences the affinity of the agonist, then the dissociation constant of the allosteric ligand can be calculated from the results of experiments in which the concentration of agonist required for half-maximal response (EC_{50}) is measured in the presence of various concentrations of the allosteric ligand. If the agonist used in the pharmacological experiments is a partial agonist with little receptor reserve, then the shift in the EC_{50} of the agonist caused by the allosteric ligand may be dependent on the affinity of the agonist.

Receptor theory provides a means of estimating the dissociation constants of agonists and antagonists from pharmacological data. This theory rests on the assumption that the response of the tissue is some function of the stimulus, with the stimulus being equivalent to the product of intrinsic efficacy and receptor occupancy. The influence of the stimulus-response function on the estimate of the dissociation constant can be eliminated by examining the relationship between equal tissue responses. Null methods which utilize this approach have been developed for estimating the dissociation constants of agonists and antagonists. These methods include determining the dissociation constants of: 1) partial agonists, by comparing their responses with equivalent responses of a more efficacious agonist (1, 2); 2) agonists, by comparing equal tissue responses before and after partial receptor inactivation (3); and 3) competitive antagonists, by comparing equal tissue responses to an agonist in the presence and absence of the antagonist (4).

In principle, it should also be possible to use a pharmacological procedure to estimate the dissociation constant of a drug which interacts allosterically with a receptor to modify the response to an agonist. Such a drug should only affect the affinity and efficacy components of the agonist without influencing the stimulus-response function; consequently, a null method based on comparing equal tissue responses to an agonist in the presence and absence of an allosteric ligand would be justified. In the present report, the theoretical basis for such a method is described as well as some theoretical examples. The results of this analysis show that the concentration of a negative allosteric ligand required to shift the agonist dose-response curve 2-fold is often a good approximation of the dissociation constant of the allosteric drug for its site on the receptor.

A necessary part of this analysis includes the mathematical derivation of allosteric interactions between drugs which bind at distinct sites on the same receptor. These mathematics should have practical application to the analysis of radioligand binding experiments in which the influence of allosteric ligands on the binding of a radioligand is measured. Consequently, methods for estimating the affinity and cooperativity of allosteric ligands using radioligand methods are also described. Part of the radioligand binding analysis has been described by Stockton *et al.* (5).

Theory

Analysis of Allosteric Interactions in Radioligand Binding Experiments

The scheme which describes the interaction between two drugs (X and A) that bind at distinct sites on the same receptor complex is:

$$\begin{array}{ccc} & K_X & \\ [X[+ [R] + [A] & \rightleftharpoons & [XR] + [A] \\ K_A \;\Big\Downarrow & & \Big\Downarrow\; \alpha K_A \\ [X] + [RA] & \rightleftharpoons & [XRA] \\ & \alpha K_X & \end{array}$$

In this scheme, K_X denotes the dissociation constant of radioligand X for its site on the receptor (R), K_A denotes the dissociation constant of A for its site, and XR and AR represent the drug-receptor complexes. It can be shown that, at equilibrium, the effect which X has on the affinity of A is equivalent to the effect which A has on the affinity of X (6). This reciprocal interaction is called cooperativity, and its magnitude is equivalent to the factor (α) by which the two drugs either increase or decrease their respective dissociation constants. Thus, αK_A denotes the dissociation constant for the binding of A to XR, αK_X denotes the dissociation constant for the binding of X to RA, and XRA is the resultant ternary complex. The equation which describes the binding of X in the presence of A is derived in the Appendix (Eq. A10) and is given below:

$$Y = \frac{[X][R_T]}{[X] + K_X'} \quad (1)$$

where:

$$K_X' = K_X \times \left(\frac{K_A + [A]}{K_A + [A]/\alpha}\right)$$

In this equation, Y denotes receptor-bound X ($Y = XR + XRA$), K_X' denotes the apparent dissociation constant of X, and R_T denotes the total receptor concentration.

It is informative to see how the theoretical occupancy curve of X changes in the presence of increasing amounts of A. In Fig. 1A, the theoretical occupancy curve for X is calculated in the presence of increasing concentrations of A. For this example, it was assumed that the two drugs inhibited the binding of each other or, in other words, the two drugs displayed negative heterotropic cooperativity. The magnitude of this cooperativity was assigned a value of 10 (i.e., $\alpha = 10$). It can be seen in Fig. 1A that the occupancy curve for X shifts to the right in the presence of increasing concentrations of A, but there is a limit to the shift. Fig. 1B shows a Scatchard plot of the data. It is apparent that the binding of X in both the presence and absence of A is consistent with a simple one-site model, resulting in a linear Scatchard plot, and that the effect of A is to produce a competitive-like reduction in the slope of the plot (decrease in affinity). It can be shown that the slope of the Scatchard plot is equivalent to $-1/K_X'$. Thus, it will be impossible to distinguish this negative heterotropic cooperativity from competitive inhibition if an experiment is run in the presence of only one concentration of A. The effects of various concentrations of A on K_X' are shown graphically in Fig. 1C. Here log ($K_X'/K_X - 1$) is plotted against log A in a manner analogous to a Schild plot. It can be seen that, unlike competitive inhibition, the maximum inhibition caused by the allosteric ligand A reaches a plateau at $K_X'/K_X = \alpha$. As described above, the apparent dissociation constant of X is equivalent to:

$$K_X' = K_X \times \left(\frac{K_A + [A]}{K_A + [A]/\alpha}\right) \quad (2)$$

When the negative cooperativity is very great (i.e., $A/K_A \ll \alpha$), then:

$$K_X' \cong K_X(1 + [A]/K_A) \quad (3)$$

and the nature of the inhibition caused by A will resemble competitive antagonism. This similarity with competitive inhibition is seen in Fig. 1C by that portion of the curve which is roughly parallel with the *dashed line*. It can also be seen that

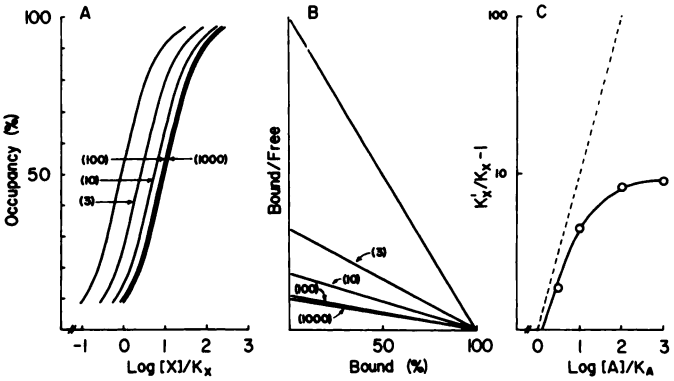

Fig. 1. Effects of a negative allosteric ligand, *A*, on the binding of radioligand, *X*. The *numbers in parentheses* refer to the relative concentration of *A* ($[A]/K_A$). A. Receptor occupancy for radioligand *X* is plotted against the relative concentration of *X* ($[X]/K_X$), in the absence and presence of various concentrations of the allosteric ligand *A*. The theoretical curves were calculated according to Eq. 1 assuming $\alpha = 10$. *Occupancy* is expressed as percentage and is equivalent to $100 \times ([XR] + [XRA])/[R_T]$. B. Scatchard plot of the data shown in A. *Bound* denotes receptor occupancy by *X*, and *Free* denotes the concentration of *X*. C. Schild plot of the data shown in A. – – –, the expected behavior for competitive antagonism.

the concentration of A required to cause a 2-fold increase in K_X' is approximately equal to K_A. Thus, the pA_2 value (X intercept) is a reasonable estimate of the negative log of the dissociation constant of the allosteric ligand if the negative cooperativity is great (i.e., $\alpha > 10$). However, it is often stated that the pA_2 value is only an estimate of $-\log K_A$ if A is a competitive antagonist. In order to obtain a more precise estimate of K_A, the data in Fig. 1 can be analyzed by nonlinear regression analysis according to the log form of Eq. 2:

$$\log K_X' = \log\left(K_X \times \frac{K_A + [A]}{K_A + [A]/\alpha}\right) \quad (4)$$

In regression analysis, the estimates of log K_X' are regressed against the independent variable A. This analysis will provide estimates of K_A, K_X, and α. The log form of the equation is used since the estimates of K_X' have a more uniform variance when expressed as a log.

The consequences of positive heterotropic cooperativity represent the converse of the results shown in Fig. 1. In this case (where $\alpha < 1$), the allosteric ligand causes a concentration-dependent enhancement of the binding of X as shown in Fig. 2. For this example, it was assumed that the positive cooperativity was equivalent to 0.1. It can be seen in Fig. 2A that, in the presence of increasing concentrations of A, the occupancy curve of X shifts to the left. The Scatchard plots in Fig. 2B show that the allosteric effects of A are entirely attributable to a selective reduction in the dissociation constant of X without an effect on binding capacity. It is convenient to express the positive heterotropic effects of A according to the following equation, which is simply a rearrangement of Eq. 2:

$$\frac{K_X}{K_X'} = \frac{K_A + [A]/\alpha}{K_A + [A]} \quad (5)$$

In Fig. 2C the effect of A on the apparent dissociation constant of X is plotted against log A according the equation described above. At high concentrations of A, the value of K_X/K_X' reaches a plateau of $1/\alpha$ (10 in this example), which is the maximum increase in affinity. It can be shown from Eq. 5 that K_A is

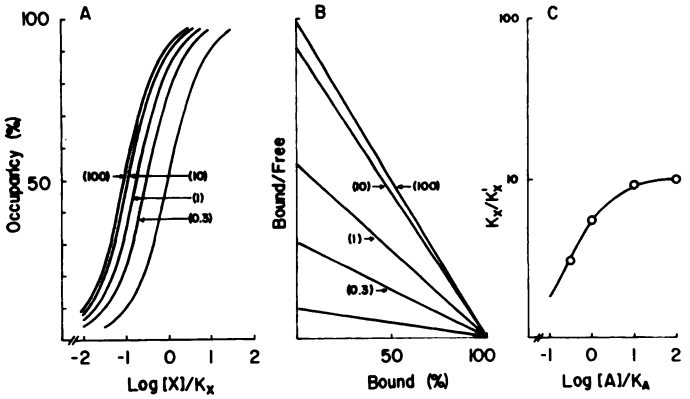

Fig. 2. Effects of a positive allosteric ligand, *A*, on the binding of radioligand, *X*. The *numbers in parentheses* refer to the relative concentration of *A* ($[A]/K_A$). A. Receptor occupancy for radioligand *X* is plotted against the relative concentration of *X* ($[X]/K_X$), in the absence and presence of various concentrations of the allosteric ligand *A*. The theoretical curves were calculated according to Eq. 1 assuming $\alpha = 0.1$. *Occupancy* is expressed as percentage and is equivalent to $100 \times ([XR] + [XRA])/[R_T]$. B. A Scatchard plot of the data shown in A. *Bound* denotes receptor occupancy by *X*, and *Free* denotes the concentration of *X*. C. The ratio of K_X/K_X' is plotted against log [*A*]. The data are from Fig. 2A.

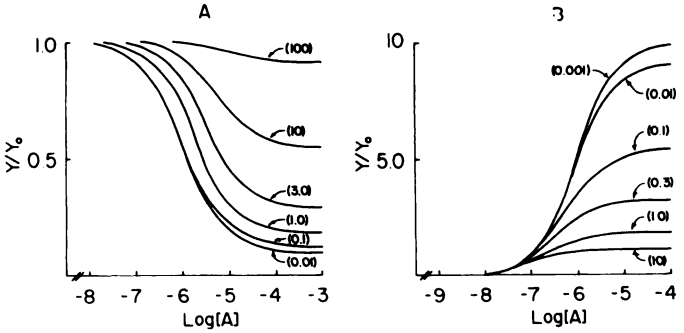

Fig. 3. Allosteric effects of *A* on the binding of radioligand *X* at a fixed concentration. Each curve represents the fractional binding of *X* (Y/Y_0) at a fixed concentration, plotted against log [*A*]. The *numbers in parentheses* denote the relative concentration of *X* ($[X]/K_X$) that was used for each curve. The theoretical curves were calculated using Eq. 6. A. Effects of a negative allosteric ligand, *A*, on the binding of radioligand, *X*. For the calculation of the theoretical curves, $\alpha = 10$ and $K_A = 1.0$ μM. B. Effects of a positive allosteric ligand, *A*, on the binding of radioligand, *X*. For the calculation of the theoretical curves, $\alpha = 0.1$ and $K_A = 1.0$ μM.

equivalent to the concentration of *A* which yields a K_X/K_X' ratio of $1/2 \times (1 + 1/\alpha)$ (5.5 in this example). The consequences of Eq. 5 also predict that when the positive cooperativity is very great ($\alpha \ll 1$), the concentration of *A* yielding a 2-fold reduction in K_X' is approximately equal to αK_A. A precise estimate of K_A can be obtained by fitting Eq. 4 to the data in Fig. 2C by nonlinear regression analysis.

It is often more economical to estimate the binding parameters of a nonlabeled drug by measuring the binding of a fixed concentration of a radioligand in the presence of various concentrations of the nonlabeled drug. The equation for the fractional binding of *X* at a fixed concentration in the presence of various concentrations of *A* is:

$$\frac{Y}{Y_0} = \frac{[X] + K_X}{[X] + K_X'} \tag{6}$$

In this equation, K_X' is defined as it is in Eq. 2, and Y_0 denotes the binding of *X* in the absence of *A*. The derivation of this equation is given in the Appendix (see Eq. A13). Fig. 3 shows the theoretical binding curves for a ligand which allosterically inhibits the binding of *X* (Fig. 3A) and a ligand which allosterically enhances the binding of *X* (Fig. 3B). For these examples, the magnitude of the negative and positive cooperativity (α) was assigned values of 10 and 0.1, respectively. In the case of negative heterotropic cooperativity (Fig. 3A), the maximum inhibition caused by *A*, does not reach 100% like that of a competitive antagonist, but, rather, the inhibition curve reaches a plateau at some measurable level of binding. Moreover, increasing the concentration of the radioligand causes a shift to the right and a reduction in the maximum inhibition of binding. The consequences of positive heterotropic cooperativity (Fig. 3B) represent the converse of the results shown in Fig. 3A. Here, the nonlabeled drug causes an enhancement of radioligand binding, and increasing the concentration of the radioligand causes a shift to the left and a reduction in the maximum of the binding curve. The relationship between the maximum effect of *A* and the estimate of the cooperativity (α) can be understood by evaluating Eq. 6 as *A* becomes infinitely large. Under these conditions Eq. 6 becomes:

$$Y' = \frac{[X] + K_X}{[X] + \alpha K_X} \tag{7}$$

in which Y' denotes the value of Y/Y_0 when *A* is infinitely large. By rearrangement:

$$\alpha = \frac{K_X + [X](1 - Y')}{K_X \times Y'} \tag{8}$$

Thus, if K_X is known, α can be calculated by substituting the estimated value of Y' into Eq. 8. It can be shown that, at very low concentrations of $[X]$ ($[X] \ll K_X$):

$$\alpha \cong \frac{1}{Y'} \tag{9}$$

The K_A value can be calculated from the graphical parameters of the plots in Fig. 2. The concentration of *A* necessary for a half-maximal effect on the binding of the radioligand (A_{50}) is related to K_A according to the following equation which is derived in the Appendix (see Eq. A18):

$$A_{50} = K_A \times \left(\frac{[X] + K_X}{[X]/\alpha + K_X}\right) \tag{10}$$

Thus, if K_X is known, and α is calculated from Eq. 8, the K_A can be estimated from the A_{50} value using the equation above. It can be shown that, at very low concentrations of *X* ($[X] \ll K_X$):

$$A_{50} \cong K_A \tag{11}$$

and, therefore, the A_{50} value will be a reasonable estimate of K_A. Also, if there is a large amount of negative cooperativity ($\alpha \gg [X]/K_X$), then:

$$A_{50} \cong K_A\,(1 + [X]/K_X) \tag{12}$$

Consequently, under these conditions, the relationship between A_{50} and K_A resembles the competitive relationship between the IC_{50} and K_i of a nonlabeled competitive inhibitor [see Chang and Prusoff (7)].

Table 1 lists the estimates of the A_{50} values for the theoretical data in Fig. 3A, together with the approximate estimates of K_A

TABLE 1

Effects of a negative allosteric ligand, *A*, on the binding of radioligand *X*[a]

$[X]/K_x$	A_{50}	$K_{A\text{-corr}}$[b]	Y'	$1/Y'$	Maximum inhibition[c] (%)
	μM	μM			%
0.01	1.01	0.999	0.101	9.9	89.9
0.1	1.09	0.99	0.11	9.2	89
1.0	1.82	0.91	0.18	5.5	82
3.0	3.08	0.77	0.29	3.5	71
10.0	5.50	0.50	0.55	1.8	45
100.0	9.18	0.091	0.92	1.1	8.2

[a] The estimates listed in the table are calculated from the data shown in Fig. 3A. For this example, K_A = 1.0 μM and α = 10.
[b] Approximate estimate of K_A calculated by Eq. 12.
[c] Maximum percentage of inhibition of the binding of radioligand *X* caused by high concentrations of *A*.

calculated by Eq. 12. At low concentrations of X, it is apparent that the approximate, competitive-like correction factor (Eq. 12) gives a reasonable estimate of K_A. However, as the concentration of X increases such that $X/K_X > \alpha$, then the deviations between the true estimate of K_X and the calculated estimate become large. Table 1 also lists the estimates of Y' and $1/Y'$ for the various curves shown in Fig. 3A. It can be seen that at the lowest concentration of X, $1/Y'$ is a reasonable estimate of the negative cooperativity (α = 10). For each pair of A_{50} and Y' values listed in Table 1, it is possible to calculate the same true estimates of K_A and α (1.0 μM and 10, respectively) by application of Eqs. 8 and 10.

The most precise method for estimating both the K_A and α values from data like those shown in Fig. 3 is to use nonlinear regression analysis to obtain the estimates of K_A and α which give the least squares fit of Eq. 6 to the data. Provided that K_X is known, estimates of K_A and α can be obtained from the data for a single binding curve.

Null Method for Estimation of K_A from Pharmacological Data

Because the relationship between receptor occupancy and the response of a tissue is variable and may differ greatly from proportionality, it will be necessary to establish a method for estimating K_A which is independent of the stimulus-response relationship. The approach described below is a null method whereby equal tissue responses before and after treatment with the allosteric drug are compared. Beginning with the fundamental assumptions of Stephenson (8) and Furchgott (3), it can be said that the response of a tissue to agonist X is some function (f) of the stimulus, and that the stimulus is equivalent to the product of intrinsic efficacy (ϵ) and receptor occupancy (XR):

$$\text{response} = f(\epsilon \times [XR]) \tag{13}$$

In the presence of the allosteric ligand, the stimulus has two components corresponding to the two types of drug-receptor complexes (XR and XRA) that are generated. Moreover, the intrinsic efficacy of these two complexes may not be identical. Thus, in the presence of A, the response may be described as:

$$\text{response} = f(\epsilon \times [XR) + \epsilon' \times [XRA]) \tag{14}$$

where ϵ' denotes the intrinsic efficacy of the XRA complex. The equality relating equal tissue responses is:

$$f(\epsilon \times [XR]) = f(\epsilon \times [XR] + \epsilon' \times [XRA]) \tag{15}$$

where the left and right sides of the equality refer to the tissue responses in the absence and presence of A, respectively. Receptor occupancy for the control response can be substituted with the known formula, and the two components of receptor occupancy in the presence of A can be substituted with Eqs. A23 and A24 derived in the Appendix to give:

$$f\left(\epsilon \times \frac{[X]R_T}{[X] + K_X}\right) = f\left(\epsilon \times \frac{[X']R_T}{[X'] + K_X + [A][X']/\alpha K_A + [A]K_X/K_A}\right) + f\left(\epsilon' \times \frac{[X']R_T}{[X'] + \alpha K_X + \alpha[X']K_A/[A] + \alpha K_A K_X/[A]}\right) \tag{16}$$

In this equation, X and X' denote the concentrations of agonist producing equal tissue responses in the absence and presence of A. Eq. 16 reduces to:

$$\frac{[X]}{[X] + K_X} = \frac{[X']}{[X'] + K_X\,[A][X']/\alpha K_A + [A]K_X/K_A} + \frac{\epsilon'}{\epsilon} \times \frac{[X']}{[X'] + \alpha K_X + \alpha[X']K_A/[A] + \alpha K_A K_X/[A]} \tag{17}$$

Substituting C for ϵ'/ϵ and solving for X' yields:

$$[X'] = \frac{\alpha[X]K_A + \alpha[A][X]}{([A](C-1) \times [X]/K_X) + \alpha K_A + C[A]} \tag{18}$$

In its present form, Eq. 18 is cumbersome, and its practical application to pharmacological data is unclear. There are some common conditions in which Eq. 18 simplifies and is readily applicable to pharmacological data. These conditions are described below.

Estimation of the K_A of a Negative Allosteric Ligand Using a Highly Efficacious Agonist

If tissue responses to a highly efficacious agonist are measured, then often there will be a large receptor reserve ($[X]/K_X \ll 1$). Under these conditions, Eq. 18 can be approximated by the following equation:

$$[X'] = [X] \times \frac{K_A + [A]}{K_A + [A]/B} \tag{19}$$

where $B = \alpha/C$. Consequently, the relationship between equal tissue responses is independent of K_X. For measuring the K_A of a negative allosteric ligand, it is necessary to obtain dose-response data for the agonist in the presence of various concentrations of A. In principle, any level of response can be examined; however, it is more common and relevant to examine the effect of A on the EC_{50} of the agonist. Thus, Eq. 19 can be rewritten in the form:

$$EC_{50}' = EC_{50} \times \frac{K_A + [A]}{K_A + [A]/B} \tag{20}$$

in which EC_{50}' and EC_{50} denote the concentrations of agonist producing half-maximal responses in the presence and absence of A. This equation is analogous to Eq. 2 except for the replacement of the cooperativity term (α) with the term B which is a function of both the cooperativity and the change in intrinsic efficacy caused by A. In order to obtain a precise

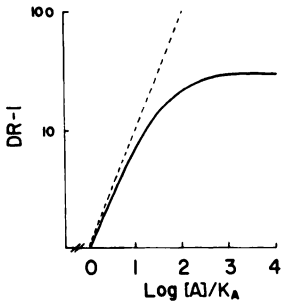

Fig. 4. Effects of a negative allosteric ligand on the EC_{50} value of an agonist with a large receptor reserve ($EC_{50}/K_X = 0.01$). The dose ratio minus one ($DR - 1$; the concentration of agonist required for half-maximal response in the presence of A divided by that measured in the absence of A, minus one) is plotted against log [A] in the form of a Schild plot. The theoretical curve was calculated according to Eq. 20, assuming $B = 30$. – – –, behavior expected for competitive antagonism.

estimate of K_A, it will be necessary to measure EC_{50} in the absence and presence of various concentrations of A. The resulting data can be analyzed according to the log form of Eq. 20 to obtain estimates of K_A and B which result in the least squares fit of the equation to the data.

The effects of a negative allosteric ligand on the dose-response curve of a highly efficacious agonist having a large receptor reserve are summarized in Fig. 4, which shows the log (dose ratio − 1) ($\log(EC_{50}'/EC_{50} - 1)$) plotted against log [$A$] in the form of a Schild plot. It can be seen that the inhibitory effect of the allosteric ligand reaches a plateau at a maximum dose-ratio equivalent to B. Thus, the maximum effect of A depends upon both the amount of negative cooperativity and the change in the intrinsic efficacy of the drug-receptor complex caused by the binding of the allosteric ligand. In general, it may not be possible to estimate α and C independently.

It is possible to employ a graphical technique to estimate the values of K_A and B for a negative allosteric ligand. As described above, the value of B can be estimated from Fig. 4 as the maximum shift in the agonist dose-response curve (i.e., EC_{50}'/EC_{50}) caused by high concentrations of the negative allosteric ligand. The value of K_A can then be calculated by rearrangement of Eq. 20:

$$K_A = \frac{[A]}{DR - 1} \times \left(1 - \frac{DR}{B}\right) \tag{21}$$

in which DR denotes the dose ratio (EC_{50}'/EC_{50}). Thus, if B is estimated graphically, the value of K_A can be calculated from data obtained at a submaximal dose ratio by substituting the appropriate values of [A] and DR into Eq. 21.

When the value of B is large with respect to the measured dose ratio, Eq. 21 can be approximated by:

$$K_A = \frac{[A]}{DR - 1} \tag{22}$$

Under these conditions, the inhibition will resemble competitive antagonism, and the concentration of the negative allosteric ligand causing a 2-fold increase in EC_{50}' will be a good approximation of K_A.

It is apparent from the foregoing that allosteric antagonism of the responses of a highly efficacious agonist is analogous to negative heterotropic cooperativity in receptor binding (see Fig. 1). The principle difference is that, in receptor binding, the maximum effect of the allosteric ligand is determined solely by the cooperativity, whereas in antagonism of tissue responses, the maximum effect depends on both the cooperativity and the change in intrinsic efficacy of the ternary complex.

Estimation of the K_A of a Negative Allosteric Ligand which Only Influences the Affinity of the Agonist

If the allosteric ligand only affects the affinity of the drug-receptor complex without influencing the intrinsic efficacy of the agonist, then Eq. 18 can be simplified by evaluating it at $C = 1$:

$$[X'] = [X] \times \frac{K_A + [A]}{K_A + [A]/\alpha} \tag{23}$$

and the K_A can be estimated as described in the previous section, even if the agonist is a partial agonist ($EC_{50}/K_X \cong 1$).

In many instances, the magnitude of the receptor reserve for the agonist may not be known, and it might not be possible to determine whether the allosteric ligand has an effect on intrinsic efficacy. Nevertheless, it should be possible to determine whether Eqs. 20 and 23 are applicable. If the negative allosteric ligand causes a change in the slope of the agonist dose-response curve at relatively low dose ratios (e.g $EC_{50}'/EC_{50} < 10$), then Eqs. 20 and 23 are not applicable. A change in slope would occur with an agonist having little receptor reserve if the allosteric ligand has an effect on intrinsic efficacy. It is appropriate to use Eq. 20 if the allosteric ligand only influences the slope of the dose-response curve at relatively large dose ratios (e.g., $EC_{50}'/EC_{50} > 10$). This behavior might be expected if responses to an agonist having a large receptor reserve are measured in the presence of high concentrations of a negative allosteric ligand which influences the intrinsic efficacy of the agonist. If the negative allosteric ligand shifts the agonist dose-response curve without affecting its slope, and it is known that the agonist has little or no receptor reserve, then it can be concluded that the negative allosteric ligand only affects the affinity of the agonist; consequently, Eq. 23 is applicable.

Estimation of the K_A of a Noncompetitive Antagonist Using a Highly Efficacious Agonist

It is possible that the allosteric ligand may bind to the receptor at a secondary site and have no effect on the binding of the agonist to the primary site (i.e., $\alpha = 1$), but may cause a decrease in the intrinsic efficacy of the drug receptor complex ($C < 1$). This condition is analogous to noncompetitive inhibition in enzyme kinetics (9). If the noncompetitive inhibitor is used against an agonist having a large receptor reserve, then Eq. 19 reduces to:

$$EC_{50}' = EC_{50} \times \frac{K_A + [A]}{K_A + C[A]} \tag{24}$$

Under these conditions, the equation is of the same form as Eq. 20, and the K_A can be estimated as described above. Here the maximum dose ratio caused by the noncompetitive antagonist is equivalent to $1/C$.

Estimation of the K_A of a Positive Allosteric Ligand

It is also possible to estimate the dissociation constant of a drug that binds at a secondary site to enhance the response of the agonist that binds at the primary site. Provided that the allosteric ligand (A) produces no response in the absence of agonist (X) and that the agonist has a large receptor reserve, then Eq. 20 is applicable. To estimate the K_A of the positive

allosteric ligand, it is necessary to obtain dose-response curves for the agonist in the absence and presence of various concentrations of A to determine the effect of A on the EC_{50} value of X. By fitting the log form of Eq. 20 to the data by nonlinear regression analysis, it should be possible to obtain estimates of K_A and B.

It is informative to rearrange Eq. 20 to the form shown below, so that the relationship between the pharmacological parameters (K_A and B) and the data is apparent.

$$\frac{EC_{50}}{EC_{50}'} = \frac{K_A + [A]/B}{K_A + [A]} \quad (25)$$

In this equation, EC_{50} and EC_{50}' are the concentrations of agonist producing half-maximal response in the absence and presence of A. When the concentration of A is large, then the ratio EC_{50}/EC_{50}' reaches a maximum, equivalent to B. Also, when A is present at a concentration equivalent to K_A, then the EC_{50}/EC_{50}' ratio is equal to $0.5(1 + B)$. Moreover, when there is a great amount of positive cooperativity (i.e., $\alpha \ll 1$), then the concentration of A resulting in a 2-fold increase in the EC_{50}/EC_{50}' ratio is approximately equal to αK_A. Thus, the effects of a positive allosteric ligand on the responses of a highly efficacious agonist are akin to positive heterotropic cooperativity in receptor binding (see Fig. 2, Eq. 5), the principle difference being that, in receptor binding, the maximum increase in affinity caused by A is determined solely by the cooperativity, whereas in enhancement of pharmacological responses, the maximum effect of A is dependent upon the cooperativity and the change in intrinsic efficacy.

The consequences of Eq. 20 also predict that it is possible to estimate the K_A of a positive allosteric ligand by measuring its potentiation of responses of an agonist having little receptor reserve if the allosteric ligand selectively enhances the affinity of the agonist and has no influence on its intrinsic efficacy. Furthermore, it is possible to estimate the K_A of an allosteric ligand which selectively increases the intrinsic efficacy of an agonist without influencing the affinity, provided that the agonist used in the pharmacological experiments has a large receptor reserve (i.e., $EC_{50}/K_X \ll 1$). The rationale and methods of analysis are analogous to those described above for negative allosteric ligands.

Conclusions

The occurrence of allosteric interactions between linked binding sites is widespread in biology. Some examples of binding sites for which specific allosteric interactions have been described include the γ-aminobutyric acid receptor-benzodiazepine receptor-chloride channel complex [see review by Olsen (10)], muscarinic receptors (5), calcium channels (11), and numerous receptors that are allosterically influenced by GTP via a guanine nucleotide-binding protein. Drugs that bind at sites linked to the primary recognition sites for endogenous neurotransmitters have the potential to enhance or inhibit ongoing neurotransmission. This type of modulation has some advantages over that which is achieved with drugs which interact directly with the primary recognition site for neurotransmitters [see Ehlert (12)].

Receptor binding studies can be used to estimate the affinity of a ligand which binds at a secondary site linked to the primary site at which the radioligand binds. The mathematics describing the influence of an allosteric ligand on the apparent dissociation constant of a radioligand and on the binding of a fixed concentration of the radioligand have been described by Stockton *et al.* (5). Eqs. 1 and 6 in the present paper are equivalent to those described by Stockton *et al.* (5). The present analysis also describes how the binding parameters are related in a simple way to graphs of the radioligand binding data. For example, it was shown that the concentration of allosteric ligand which produces half of its proximal effect on the binding of a fixed concentration of a radioligand (A_{50}) is a reasonable estimate of its dissociation constant provided that the concentration of the radioligand is less than its dissociation constant. Moreover, the concentration of a negative allosteric ligand which produces a 2-fold increase in the apparent dissociation constant of a radioligand is often a good estimate of its dissociation constant if the negative cooperativity is large (i.e., $\alpha > 10$).

In many instances the modulatory effects of an allosteric ligand on the pharmacological responses to an agonist are analogous to those seen in radioligand binding experiments. However, there is the important difference, that the effects seen in radioligand binding experiments can be attributed to changes in affinity, whereas those seen in pharmacological experiments may be due to changes in both affinity and intrinsic efficacy. In practice it may not be possible to determine whether the allosteric drug is affecting both affinity and intrinsic efficacy or affecting only one or the other. This does not introduce any problems in the estimate of the dissociation constant provided that the agonist used in the analysis is highly efficacious with a large receptor reserve. However, if the agonist has no receptor reserve, and the allosteric ligand influences the intrinsic efficacy of the agonist, then the analysis is more complicated and would require an estimate of the dissociation constant of the agonist (see Eq. 18). It can be said as a general rule that, if a negative allosteric ligand has no effect on the slope of the agonist dose-response curve at dose ratios less than 10, then the estimate of the dissociation constant of the allosteric ligand is independent of the dissociation constant of the agonist and can be calculated as described above. The same rule applies to a positive allosteric ligand provided that it does not affect the slope of the agonist dose-response curve at dose ratios greater than 0.1.

For competitive antagonism, the shift in the agonist dose-response curve (dose ratio) caused by a given concentration of the antagonist is independent of the agonist. In contrast, the magnitude of the dose ratio caused by a certain concentration of a negative allosteric ligand may vary with different agonists because the magnitude of the negative cooperativity (α) or change in intrinsic efficacy (C) is not constant for a given negative allosteric ligand, but, rather, it depends on the particular agonist with which it is interacting. Nevertheless, when estimated by the methods described in this paper, the estimate of the dissociation constant of the negative allosteric ligand should be independent of the agonist. Moreover, if the negative cooperativity between the binding of the agonist and the allosteric ligand is great, then the pA_2 value of the Schild plot will be independent of the agonist and a good estimate of the negative log of the dissociation constant of the negative allosteric ligand. In this connection, Clark and Mitchelson (13) have examined the influence of gallamine on the negative chronotropic and inotropic responses of carbachol and acetylcholine in the isolated atria and have found that gallamine produces an allosteric antagonism of these responses. They also observed that, when present at high concentrations, gallamine produced different shifts in the dose-response curves of acetylcholine and carbachol, yet, nevertheless, at low concentrations

gallamine caused similar dose ratios for the two agonists, and the same pA_2 value was calculated for gallamine from the Schild plot of the two sets of data.

The analysis described herein shows that the radioligand binding methods and pharmacological null methods, which have been developed and implemented primarily for examining competitive interactions, can also be applied quantitatively to examine allosteric interactions. As the number of compounds known to have allosteric effects on receptor sites increases, methods for analysis of allosteric interactions will have more application in basic pharmacological research.

Appendix

The derivation of the various equations shown in this paper is described here. These equations are based on the following model which describes the interactions between a radioligand or agonist (X) and another ligand (A) which bind at separate, but linked, binding sites on the same receptor complex (R):

$$\begin{array}{ccc} & K_X & \\ X + R + A & \rightleftharpoons & XR + A \\ K_A \;\Updownarrow & & \Updownarrow \;\alpha K_A \\ X + RA & \rightleftharpoons & XRA \\ & \alpha K_X & \end{array}$$

In this scheme, XR and RA denote the drug-receptor complexes, and XRA denotes the ternary complex. The dissociation constants are defined as:

$$K_X = \frac{[X][R]}{[XR]} \quad \text{(A1)}$$

$$K_A = \frac{[R][A]}{[RA]} \quad \text{(A2)}$$

$$\alpha K_X = \frac{[X][RA]}{[XRA]} \quad \text{(A3)}$$

$$\alpha K_A = \frac{[XR][A]}{[XRA]} \quad \text{(A4)}$$

The following substitution is useful:

$$[R_T] = [R] + [XR] + [RA] + [XRA] \quad \text{(A5)}$$

The Binding of X in the Presence of A

To derive the equation describing the binding of X in the presence of A, we begin with:

$$\frac{[XR] + [XRA]}{[R_T]} = \frac{[XR] + [XRA]}{[R] + [XR] + [RA] + [XRA]} \quad \text{(A6)}$$

Multiplying the numerator and denominator of the fraction on the right side of the equation by $1/[X][R]$) yields:

$$\frac{[XR] + [XRA]}{[R_T]} = \frac{\frac{[XR]}{[X][R]} + \frac{[XRA]}{[X][R]}}{\frac{[R]}{[X][R]} + \frac{[XR]}{[X][R]} + \frac{[RA]}{[X][R]} + \frac{[XRA]}{[X][R]}} \quad \text{(A7)}$$

Making the appropriate substitutions yields:

$$\frac{[XR] + [XRA]}{[R_T]} = \frac{\frac{1}{K_X} + \frac{[XRA]}{[X][R]}}{\frac{1}{K_X} + \frac{1}{[X]} + \frac{[XRA]}{[X][R]} + \frac{[RA]}{[X][R]}} \quad \text{(A8)}$$

Making the following substitutions:

$$[XRA] = \frac{[XR][A]}{\alpha K_A}; \qquad [R] = \frac{K_A[RA]}{[A]}$$

yields:

$$\frac{[XR] + [XRA]}{[R_T]} = \frac{\frac{1}{K_X} + \frac{[A]}{\alpha K_X K_A}}{\frac{1}{K_X} + \frac{1}{[X]} + \frac{[A]}{\alpha K_A K_X} + \frac{[A]}{[X]K_A}} \quad \text{(A9)}$$

This equation simplifies to:

$$Y = \frac{[X][R_T]}{[X] + K_X'} \quad \text{(A10)}$$

where:

$$K_X' = K_X \times \frac{K_A + [A]}{K_A + [A]/\alpha}$$

In this equation, Y denotes the sum of $[XR] + [XRA]$.

The Fractional Binding of X in the Presence of A

To determine the equation describing the fractional binding of X at a fixed concentration in the presence of various concentrations of A, we begin with:

$$\frac{Y}{Y_0} = \frac{[XR][XRA]}{[XR]} \quad \text{(A11)}$$

in which Y and Y_0 denote the binding of X in the presence and absence of A. By applying Eq. A10 it can be shown that:

$$\frac{Y}{Y_0} = \frac{\frac{[X][R_T]}{[X] + K_X'}}{\frac{[X][R_T]}{[X] + K_X}} \quad \text{(A12)}$$

This equation reduces to:

$$\frac{Y}{Y_0} = \frac{[X] + K_X}{[X] + K_X'} \quad \text{(A13)}$$

Estimation of the A_{50} of an Allosteric Ligand

The concentration of a negative allosteric ligand (for which $\alpha > 1$) causing half of its maximal effect on the binding of X (A_{50}) is described by the following equation:

$$1 - g(A_{50}) = \frac{1}{2} \times (1 - Y') \quad \text{(A14)}$$

In this equation, $g(A)$ denotes the function describing the fractional binding of X in the presence of A, and Y' denotes the fractional binding of X in the presence of a maximally effective concentration of A. By making the appropriate substitution for K_X' into Eq. A13, it can be shown that:

$$g(A) = \frac{Y}{Y_0} = \frac{[X]K_A + K_AK_X + [A][X]/\alpha + [A]K_X/\alpha}{[X]K_A + K_AK_X + [A][X]/\alpha + [A]K_X} \quad \text{(A15)}$$

Evaluating $g(A)$ at an infinitely large value of A yields:

$$Y' = \frac{[X] + K_X}{[X] + \alpha K_X} \quad \text{(A16)}$$

In which Y' denotes the value of Y/Y_0 in the presence of an infinitely large concentration of A. Substituting Eqs. A15 and A16 into Eq. A14 yields:

$$1-\frac{[X]K_A+K_AK_X+[A_{50}][X]/\alpha+[A_{50}]K_X/\alpha}{[X]K_A+K_AK_X+[A_{50}][X]/\alpha+[A_{50}]K_X}$$

$$=\frac{1}{2}\times\left(1-\frac{[X]+K_X}{[X]+\alpha K_X}\right) \quad \text{(A17)}$$

Solving for A_{50} yields:

$$A_{50}=K_A\times\frac{K_X+[X]}{K_X+[X]/\alpha} \quad \text{(A18)}$$

The concentration of a positive allosteric ligand (for which $\alpha<1$) causing half of its maximal effect on the binding of X (A_{50}) can be described by the following equation:

$$g(A_{50})-1=\frac{1}{2}\times(Y'-1) \quad \text{(A19)}$$

By making the appropriate substitutions for $g(A_{50})$ and Y', it can be shown that the equation describing the A_{50} value of a positive allosteric ligand is equivalent to Eq. A18.

The Proportion of Agonist Bound in the Form of *XR* and *XRA* in the Presence of *A*

To derive an equation describing the proportion of agonist X bound in the form of XR in the presence of A, we begin with the following equation:

$$\frac{[XR]}{[R_T]}=\frac{[XR]}{[R]+[XR]+[RA]+[XRA]} \quad \text{(A20)}$$

Using strategy similar to that applied in Eqs. A7–A10:

$$\frac{[XR]}{[R_T]}=\frac{\frac{[XR]}{[X'][R]}}{\frac{[R]}{[X'][R]}+\frac{[XR]}{[X'][R]}+\frac{[RA]}{[X'][R]}+\frac{[XRA]}{[X'][R]}} \quad \text{(A21)}$$

$$\frac{[XR]}{[R_T]}=\frac{\frac{1}{K_X}}{\frac{1}{K_X}+\frac{1}{[X']}+\frac{[A]}{\alpha K_AK_X}+\frac{[A]}{[X']K_A}} \quad \text{(A22)}$$

$$[XR]=\frac{[X'][R_T]}{[X']+K_X+[A][X']/\alpha K_A+[A]K_X/K_A} \quad \text{(A23)}$$

Using strategy similar to that applied in Eqs. A20–A23, it can be shown that the equation describing the amount of agonist bound in the form of XRA is:

$$[XRA]=\frac{[X'][R_T]}{[X']+\alpha K_X+\alpha[X']K_A/[A]+\alpha K_AK_X/[A]} \quad \text{(A24)}$$

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