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New methods for determining dissociation constants of agonist-receptor complexes

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Abstract

Methods are presented which enables the dissociation constant of agonist-receptor complexes (K_A) to be estimated without knowledge of the relative intrinsic efficacies. The dissociation constants are estimated from fixed agonist-competitive antagonist concentration combinations which simulate partial agonists. In general, estimating of K_A by employing these methods require ϕ and K_B to be known; where ϕ = [antagonist]/[agonist] and K_B is the dissociation constant of the competitive antagonist-receptor complex. Some of these methods require only knowledge of ϕ, while K_B may be unknown. A method is also described to estimate K_A without knowing the value of K_B and the competitive antagonist concentration. In addition to the estimation of K_A values, a new method for estimation of the dissociation constants of competitive antagonist-receptor complexes (K_B) is reported. One of the new methods was exemplified practically by using sets of experimental agonist concentration-effect curves determined in the absence and presence of increasing concentrations of competitive antagonists. From this pharmacological data the apparent K_A value for carbachol (in muscarinic M_3 receptors of the guinea-pig ileum) was determined. This action illustrated that the apparent affinity values (K_A) and height of the stimulus curve of an agonist may be determined from published pharmacological data. This procedure affords a possibility to establish whether or not spare receptors are present in a particular system. It was also shown that relative affinity values provide more reliable information than does isolated K_A values.

Keywords: Agonist affinity; Agonist-antagonist combination, fixed; Carbachol; Tripitramine; Spare receptor; (New methods)

1. Introduction

The aim of the present paper is to set out in detail the basis of new methods for estimating apparent dissociation constants (K_A) of agonist-receptor complexes, because the methods currently available often are not accurate enough to estimate and quantify subtle differences in dissociation constants. Much effort has been expended in the measurement, quantification, and comparison of agonist affinity for the classification of drugs and drug receptors because the equilibrium dissociation constant for an agonist-receptor complex, being a chemical term, can be used to classify drugs and receptors.

Since 1956 various researchers sought methods for determining dissociation constants of agonist-receptor complexes and intrinsic efficacy of agonists when a non-linear stimulus-effect coupling is present in an effector. The most promising methods for the determination of these constants are based on the so-called null method. The null method is based on the hypothesis that equal stimuli produce equal responses from any single piece of isolated tissue, and leads to null equations which relate those concentrations of drugs that produce equal responses (for references see: Mackay, 1977 and Kenakin, 1987b). These methods are quite elegant from a theoretical viewpoint, but unfortunately in practice they pose a number of serious problems.

The null methods currently employed to quantify agonist affinity culminate in a linear regression, which usually compare the reciprocals of equieffective agonist concentrations ($[A]_1$ and $[A]_2$), i.e. a double-reciprocal plot of the form:

$$1/[A]_1 = M/[A]_2 + C$$

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The dissociation constant of the agonist-receptor complex is usually some function of both the slope (M) and ordinate intercept (C) of the linear regression. Unfortunately this equation gives undue emphasis to small agonist concentrations, which are also subject to the greatest error. Therefore, the regression relies on data points of high error that are a considerable distance from the ordinate. The error in estimating the intercept can be so high that the regression is worthless. However, these erroneous estimates may be limited to some extend if proper weighting factors are used (Kenakin, 1987b).

Several methods employing a double-reciprocal plot have been reported. In the first instance measurements of full-agonist affinity can be made by the procedure developed by Furchgott (1966). This method is based on the comparison of concentration-effect curves obtained for an agonist A acting on the same tissue, before and after treatment of the tissue with a specific irreversible antagonist. This technique requires that a sufficient number of receptors should be irreversible inactivated so that the maximal response to the full agonist is depressed. A problem associated with this method is the assumption that the only difference produced in the tissue, by the alkylating agent, is a decrease in the number of receptors and that no change in the nature of the receptor or the coupling characteristics of the tissue have taken place. There is no proof for this prerequisite of the Furchgott method and, unfortunately, the prerequisite seems to be violated by the finding of Van Ginneken (1977). He showed that if part of the receptor pool is blocked with the irreversible antagonist dibenamine, the effect of an agonist is substantially decreased but is generated more rapidly. From this finding it was concluded, that if the effect of this irreversible noncompetitive antagonist was only blockade of part of the receptor pool, then it could hardly be expected that the rate of receptor occupation by an antagonist after application of the antagonist would change, since this rate only depends upon the magnitude of the unchangeable rate constants and of the drug concentration. Although the most common pharmacological procedure available to estimate K_A for full agonists relies upon irreversible occlusion of a fraction of the receptor population (Furchgott, 1966; Waud, 1969; Parker and Waud, 1971; Kenakin and Beek, 1984; Diaz-Toledo and Marti, 1988), it is possible that all measurements of dissociation constants for agonist-receptor complexes with this method are erroneous (Kenakin, 1989). The necessity that an irreversible antagonist is required for use of this method represents a practical problem since for most drug receptors no such tools are yet available.

A method of estimating the dissociation constant for a partial agonist-receptor complex is accomplished by using an equation derived by comparing equiactive concentrations of a full agonist A and a partial agonist P (Waud, 1969; Kenakin, 1987b). An important feature of this equation that affect its applicability for measurement of agonist affinity relates to the nature of the full and partial agonists. If one plots $1/[A]_1$ against $1/[A]_2$, then K_P, the dissociation constant of the partial agonist-receptor complex, could be obtained from the resulting straight line as follows:

$$K_P = \text{slope}/\text{intercept}(1 - \varepsilon_P/\varepsilon_A)$$

where ε_P and ε_A represent the intrinsic efficacies of the partial and full agonists respectively. In this instance there are three unknowns, namely K_P, ε_A and ε_B, which presently cannot be estimated separately from a comparison of the two curves. To overcome this obstacle, drugs are chosen so that ε_A is supposed to be considerably larger than ε_P. Under this specific condition the magnitude of the ratio $\varepsilon_P/\varepsilon_A$ is presumed to diminish to insignificance, and, if this preposition holds, it should be possible to obtain a reasonably good estimate of the dissociation constant of the partial agonist.

Stephenson (1956) reported another method for estimating the dissociation constant for a partial agonist-receptor complex by comparing equiactive concentrations of a full agonist A in the absence and presence of a partial agonist P. Again, the estimation of the dissociation constant is affected by the unknown relative efficacies of the agonists and, therefore, estimation of a dissociation constant can only be made if ε_A is considerably larger than ε_P.

The possibility of enormous errors in the present analysis of agonists, and the implications of these errors for receptor classification, create a need for a method by which more reliable dissociation constants (K_A) and/or relative affinities of agonists may be estimated. In this paper it is shown how concentration-effect curves determined with agonist-competitive antagonist combinations, consisting of fixed antagonist/agonist concentration ratios, provide such methods. One of the methods is exemplified practically and the advantages and possible limitations of the new methods are discussed.

2. Materials and methods

2.1. Theory

The new methods for estimating apparent K_A values are based on the idea that a combination consisting of a fixed concentration ratio of agonist A and competitive antagonist B will portray the general profile of a partial agonist. This agonist-antagonist combination would therefore yield a concentration-effect curve which possesses a submaximal effect.

The theoretical background of the methods is developed from the occupancy theory. It follows from the occupancy theory of agonist action that the stimulus S may be calculated by means of the following equation (Stephenson, 1956):

$$S = \frac{e}{1 + \frac{K_A}{[A]}} \qquad (1)$$

in which [A] is the concentration of agonist, e is the efficacy of agonist A and K_A is the dissociation constant for the agonist-receptor complex. Stimulus (S) may be regarded as a fraction of the maximal effect (S_A/S_m), thus $S = S_A/S_m$ where S_A represents the stimulus of agonist A and S_m represents the maximal stimulus of A (Ariëns et al., 1964). The value of efficacy (e) for a certain agonist A may arbitrary be taken as unity, because the scale of efficacy is arbitrary while the value of K_A is not influenced by efficacy. Thus, if $e = 1$, Eq. (1) can be written as:

$$S_A = \frac{S_m}{1 + \frac{K_A}{[A]}} \tag{2}$$

where the height (maximal stimulus) of the concentration-stimulus curve of agonist A is given by S_m.

Eq. (2) serves further as a basis for establishing the conditions which permit analysis of simple competitive antagonism. Simple competitive antagonism, by definition, can only affect occupancy function. Receptor theory predicts that a competitive antagonist B will affect Eq. (2) as follows (Arunlakshana and Schild, 1959; Ariëns et al., 1964):

$$S_{AB} = \frac{S_m}{1 + \left(1 + \frac{[B]}{K_B}\right)\frac{K_A}{[A]}} \tag{3}$$

in which S_{AB} signifies stimulus of agonist A in the presence of competitive antagonist B, [B] is the concentration of competitive antagonist B and K_B is the dissociation constant of the competitive antagonist-receptor complex. If [A] and [B] are combined in a fixed ratio (ϕ), then:

$$[B]/[A] = \phi \tag{4}$$

It follows from Eqs. (3) and (4) that

$$S_{AB} = \frac{S_m}{1 + \left(1 + \frac{\phi[A]}{K_B}\right)\frac{K_A}{[A]}} = \frac{S_m}{1 + \frac{\phi K_A}{K_B} + \frac{K_A}{[A]}} \tag{5}$$

Eq. (5) can be modified as follows:

$$S_{AB} = \frac{\frac{S_m}{(1 + \phi K_A/K_B)}}{1 + \left(\frac{1}{1 + \phi K_A/K_B}\right)\frac{K_A}{[A]}} \tag{6}$$

If $\phi = 0$, then Eq. (6) simplifies to Eq. (2).

Eq. (6) may be written as:

$$S_{AB} = \frac{h}{1 + \frac{[A]_{1/2}}{[A]}} \tag{7}$$

In this form Eq. (7) is seen to be Eq. (2) in which $[A]_{1/2}$ represents the agonist concentration at half-maximal ($h/2$) of the curve height (h). It follows from Eqs. (6) and (7) that:

$$[A]_{1/2} = \frac{K_A}{(1 + \phi K_A/K_B} \tag{8}$$

It follows further from Eqs. (6) and (7) that the height, h, of the concentration-stimulus curve obtained with an agonist-competitive antagonist combination is given by:

$$h = \frac{S_m}{(1 + \phi K_A/K_B)} \tag{9}$$

Eq. (8) can be rewritten as:

$$[A]_{1/2} = -\frac{K_A}{K_B}\phi[A]_{1/2} + K_A \tag{10}$$

which should result in a straight line if $[A]_{1/2}$ is plotted against $\phi[A]_{1/2}$. The K_A value of the agonist-receptor complex may be estimated from the slope ($= -K_A/K_B$) if K_B is known, or from the intercept with the ordinate ($[A]_{1/2}$ axis). The latter follows from Eq. (10), since $[A]_{1/2} = K_A$ if $\phi[A]_{1/2} = 0$. Note that $\phi[A]_{1/2} = 0$ in the absence of B, i.e. if $[B] = 0$. The intercept of the straight line with the abscissa gives the value of K_B, because, if $[A]_{1/2}$ in Eq. (10) tends to become zero, then $\phi[A]_{1/2}$ will tend to become equal to K_B. Regarding this last instance it is important to note that $[A]_{1/2} = 0$ is meaningless, therefore the value of $[A]_{1/2}$ will always be larger than zero. In practical terms this means that the value of $\phi[A]_{1/2}$ is infinite small when $[A]_{1/2}$ reaches the largest value (which is infinite small) at which the pharmacological effect is zero.

It follows from Eq. (4) that: $\phi[A]_{1/2} = [B]_{1/2}$, where $[B]_{1/2}$ represent the concentration of the competitive antagonist giving rise to $h/2$, i.e. $[B]_{1/2}$ represents the concentration of the competitive antagonist B at $[A]_{1/2}$. Eq. (10) may thus also be written as:

$$[A]_{1/2} = -\frac{K_A}{K_B}[B]_{1/2} + K_A \tag{11}$$

It is evident from Eq. (11) that a plot of $[A]_{1/2}$ on $[B]_{1/2}$ should yield a straight line, where the values of K_A and K_B are obtained by the ordinate intercept ($[A]_{1/2}$ axis) and the abscissa intercept ($[B]_{1/2}$ axis) respectively.

Analogous to Eq. (10), Eq. (9) modifies to:

$$h = -\frac{K_A}{K_B}\phi h + S_m \tag{12}$$

which also describes a linear relationship. A plot of h (heights of agonist-antagonist concentration-stimulus curves) on the product ϕh should yield a straight line. The slope of the straight line is given by $-K_A/K_B$ while the intercept with the ordinate (h axis) gives the value S_m.

Curve height h may be measured as the fraction of the maximal curve height, i.e. $h = h_{AB}/h_m$, where quantity h_{AB} represents the height of the submaximal concentration-stimulus curve(s) obtained with the fixed agonist-antagonist combination(s) and h_m represents the maximal height of the concentration-stimulus curve of the full agonist A in the absence of B. Hereafter, the height of an agonistic concentration-stimulus curve, in the absence of a competitive antagonist, will be referred to as the maximal height.

It follows from Eq. (12) that $h = S_m$ if $\phi h = 0$ (i.e. [B] = 0). Therefore, if $e = 1$ (see Eq. 2), then the value of S_m may be estimated from the intercept of the straight line with the ordinate. It should be kept in mind, however, that the value of S_m (in Eq. 12) depends on an arbitrary chosen value of curve height, h, and that S_m actually represents the maximal height of the concentration-stimulus curve of a full agonist. It is clear that the estimated value of S_m should not be regarded as an absolute value. The practical implications and possibilities of Eq. (12) will be discussed thoroughly in a following paper.

There is abundant evidence that nonlinear relationships exist for the stimulus-effect relationship of agonists. The presence of such relationships would produce dissimulations between the concentration-stimulus curves and concentration-effect curves of an agonist, and therefore agonist affinities cannot be estimated directly from concentration-effect curves. Since the new methods are especially devised for estimation of apparent K_A in systems where nonlinear relationships exist between effect and stimulus, it is assumed throughout this paper that a nonlinear stimulus-effect relationship exists in all the theoretical cases discussed (Figs. 1–4).

On the one hand there is abundant evidence pointing to a nonlinear relationship which may result in a maximal effect of an agonist on an effector before all the specific receptors are occupied by the agonist (Ruffolo, 1982). This phenomenon is referred to as receptor reserve for maximal effect (Kenakin, 1987a). On the other hand, however, it was reported that a nonlinear stimulus-effect relationship may exist in a system even though there is no receptor reserve for maximal effect. In this instance there may be a very large reserve for half-maximal effect, while maximal stimulus produces maximal effect (Kenakin, 1987a).

Because of the numerous amplification processes in cells it is not possible to equate the level of response accurately with the strength of the receptor stimulus and it is therefore impossible to establish for certainty which of the above mentioned nonlinear mechanisms will prevail in a particular agonist-receptor system. This paper focused primarily (in Section 2.4) on the study of a nonlinear stimulus-effect coupling resulting from receptor reserve for maximal response when the concentration-stimulus curves and concentration-effect curves are supposed to coincide to a certain degree. For cases where the latter possibility may prevail, it was assumed for the sake of convenience and

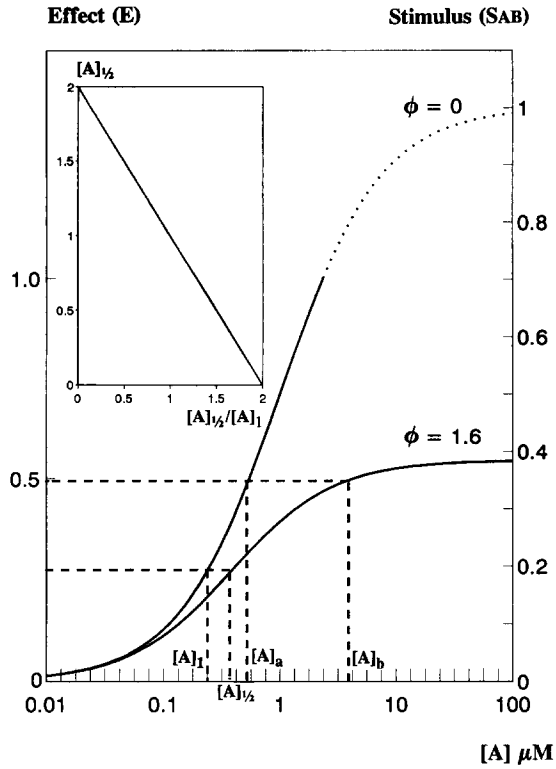

Fig. 1. Theoretical concentration-stimulus curves (dotted lines) of an agonist A calculated according to Eq. (6): $K_A = 1$ μM, $S_m = 1$, $\phi = 0$ and 1.6. The concentration-effect curves are represented as continues lines and coincide with the concentration-stimulus curves when a submaximal effect is obtained. K_B values may be estimated by employing Eq. (13) and utilizing different values for $[A]_a$ and $[A]_b$, which are obtained at the same quantitative effects. Inset: plot of $[A]_{1/2}$ against $[A]_{1/2}/[A]_1$ – Eq. (17). Intercept with ordinate: $[A]_{1/2} = 2K_A$. Intercept with abscissa: $[A]_{1/2}/[A]_1 = 2$. Slope = $-K_A$. Values of $[A]_{1/2}$ were obtained from different curves in Fig. 2 at various $[B]/[A] = \phi$ values.

simplicity that the concentration-stimulus curve and concentration-effect curve of a full agonist should partly coincide, as suggested by Van den Brink (1977) (see Figs. 1–4). It was also assumed that the sub-maximal concentration-effect curve of an agonist-competitive antagonist combination should coincide completely (Figs. 1–4). In Section 2.4 is discussed the application of the model to a nonlinear stimulus-effect relationship when a receptor reserve is present for half-maximal effect as well as maximal effect, while the stimulus and effect curves do not coincide at all (see Fig. 4).

2.2. Estimation of the dissociation constant K_B

If K_B is known, then it is evident from the foregoing theory that a K_A value can be estimated by utilizing either Eq. (10), (11) or (12). In all these procedures K_A is calculated by employing the appropriate K_B value and the slopes of resulting straight lines. The dissociation constant K_B can be estimated according to a number of methods.

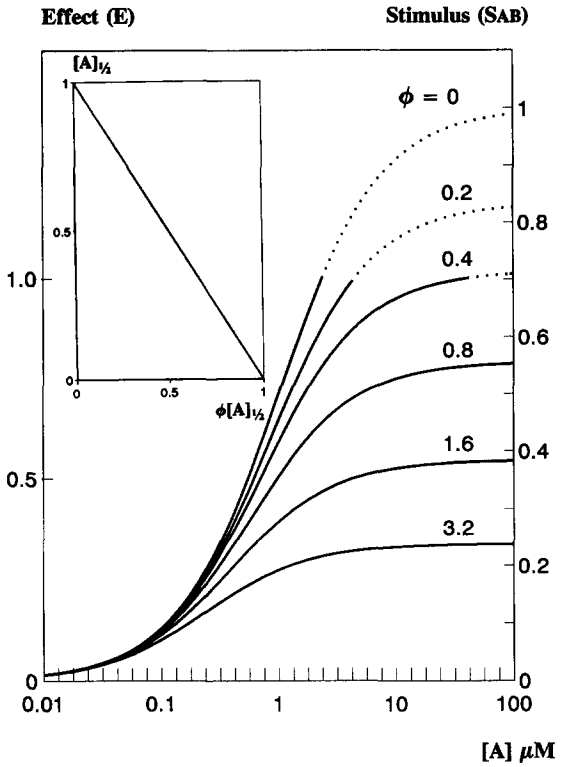

Fig. 2. Theoretical concentration-stimulus curves (dotted lines) of an agonist A calculated according to Eq. (6): $K_A = 1$ μM, $S_m = 1$, ϕ varied from 0 to 3.2). The concentration-effect curves are represented as continues lines and coincide with the concentration-stimulus curves when a sub-maximal effect is obtained, i.e. when the value of $\phi = 0.8$, 1.6 and 3.2. Curve heights (h_1, h_2, h_3) of these sub-maximal concentration-effect curves given as fractions of maximal effect ($E = 1.0$) (Table 1). Inset: plot of $[A]_{1/2}$ against $\phi[A]_{1/2}$ – Eq. (10). Intercept with ordinate: $[A]_{1/2} = K_A$. Intercept with abscissa: $\phi[A]_{1/2} = K_B$. Slope = $-K_A / K_B$.

In the first instance K_B for the purely competitive antagonist B may be estimated by the standard method for pA_2 determinations. The standard method for estimating the apparent dissociation constant of a competitive antagonist-receptor complex requires a comparison of agonist concentration-effect curves determined on effectors in the presence and absence of a range of fixed concentrations of the antagonist (Arunlakshana and Schild, 1959). The K_B value may be calculated by means of:

$$K_B = \frac{[B]}{[A]_b/[A]_a - 1} \quad (13)$$

where, in the absence of B a certain effect was caused by the agonist concentration $[A]_a$, but in its presence the same quantitative effect was caused by agonist concentration $[A]_b$.

Secondly, the K_B value may be estimated by employing fixed agonist-antagonist combinations. The concentration-effect curve of the agonist was determined alone, and thereafter a concentration-effect curve of the combined agonist-antagonist combination (constant ϕ) was determined on the same effector (see Fig. 1). The theoretical concentration-stimulus curves shown in Fig. 1 were calculated according to Eq. (6). The values of K_A, K_B and S_m were kept constant ($K_A = K_B = 1$ μM, $S_m = 1$) while the values $\phi = 0$ and $\phi = 1.6$ were used. Theoretical concentration-effect curves simulating experimental concentration-effect curves were constructed according to the assumption that the maximal effect ($E = 1$) on the effector is obtained at a stimulus value of $S_{AB} = 0.7$. By using the same method as described for the classical estimation of K_B, the K_B value may be obtained by employing Eq. (13) and data obtained from the agonist curve (when $[B] = 0$) and a single agonist-antagonist curve as shown in Fig. 1.

Thirdly, K_B may also be estimated graphically by determining concentration-effect curves of agonist-antagonist combinations for different values of the relationship $[B]/[A] = \phi$ (Fig. 2). The theoretical concentration-stimulus curves shown in Fig. 2 were calculated according to Eq. (6). The values of K_A, K_B and S_m were kept constant ($K_A = K_B = 1$ μM, $S_m = 1$) while the value for ϕ was increased from zero to 3.2. The theoretical

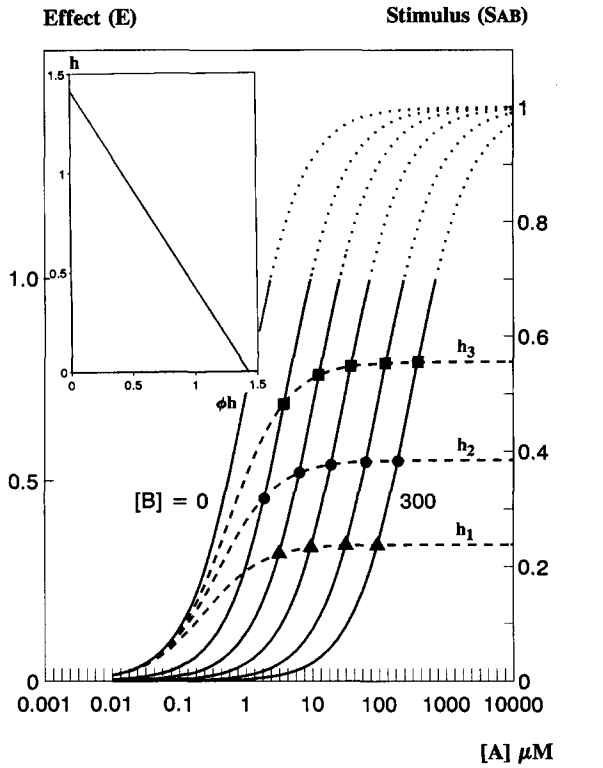

Fig. 3. Theoretical concentration-stimulus curves (dotted lines) of an agonist A, in the absence and presence of increasing concentrations of a competitive antagonist B, calculated according to Eq. (3): $K_A = K_B 1 =$ μM, $S_m = 1$, $[B] = 0$, 3, 10, 30, 100 and 300 μM. The concentration-effect curves are represented as continues lines. The broken lines represent concentration-effect curves of fixed [agonist]/[antagonist] ratios (ϕ) deduced from the effect curves (continues lines). Curve heights (h_1, h_2, h_3) (broken lines) given as fractions of maximal effect ($E = 1.0$) (Table 1). Markers represent positions of constant $\phi = [B]/[A]$ (■, $\phi = 0.8$; ●, $\phi = 1.6$; ▲, $\phi = 3.2$). Inset: plot of h against ϕh – Eq. (12). Intercept with ordinate: $h = S_m = 1.4286$. Slope = $-K_A / K_B$.

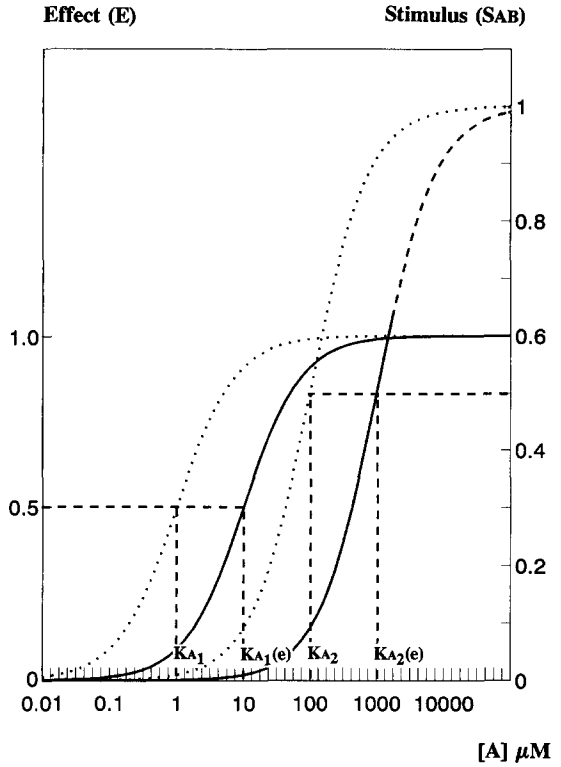

Fig. 4. Theoretical concentration-stimulus curves (dotted lines) of agonist A_1 ($K_{A_1} = 1$ μM, $e = 0.6$) and agonist A_2 ($K_{A_2} = 100$ μM, $e = 1.0$) calculated according to Eq. (1). K_{A_1} and K_{A_2} represent the actual affinities of A_1 and A_2, while $K_{A_1}(e)$ and $K_{A_2}(e)$ represent the experimental determined apparent affinities of A_1 and A_2. The concentration-effect curves of A_1 and A_2 are represented as continues lines and do not coincide with the agonistic concentration-stimulus curves (dotted lines). It was assumed that maximal effect ($E = 1$) corresponds to a stimulus value of 0.6. The S-shaped curve (broken lines) merely indicates the (apparent) position where the model expects the concentration-stimulus curve of agonist A_2 when estimating $K_{A_2}(e)$. For agonist A_1 the model assumes that the concentration-stimulus and concentration-effect curves coincide completely.

concentration-effect curves simulating experimental concentration-effect curves were constructed according to the assumption that the maximal effect ($E = 1$) is obtained at a stimulus value of $S_{AB} = 0.7$. By plotting the different values for $[A]_{1/2}$ against $\phi[A]_{1/2}$ (or $[B]_{1/2}$) a straight line was obtained as predicted by Eq. (10), or Eq. (11) (inset: Fig. 2), where the intercept of the straight line with the ordinate ($\phi[A]_{1/2}$ axis or $[B]_{1/2}$ axis) gives the dissociation constant K_B. According to Eq. (10), or Eq. (11), K_B may also be estimated from the slope and ordinate intercept of the straight line. Thus:

$$K_B = \frac{K_A}{\text{slope}} = \frac{\text{intercept of ordinate}}{\text{slope}}$$

If K_B was estimated from data shown in Figs. 1 and 2, then it would be important to ascertain whether or not the requirements of antagonist-receptor equilibrium was met during these experiments. Therefore, when possible, K_B values determined via the new and classical methods should be compared to reveal possible discrepancies between these values. Possible disparity between the K_B values determined by the different techniques may be ascribed to differences in equilibrium time allowed by the different methods. It is of course also possible to construct a Schild plot with the experimental values obtained for $([A]_b/[A]_a - 1)$ and [B] from Fig. 2. If the regression of $\log([A]_b/[A]_a - 1)$ on log[B] is linear and has a slope of unity, this furnishes presumptive evidence that the antagonism is competitive.

2.3. Estimation of agonist affinity when stimulus and effect curves coincide

Methods for the estimation of the dissociation constant K_A are discussed for the following cases: (1) when the competitive antagonist is in equilibrium with its receptors (Section 2.3.1); and (2) when the competitive antagonist is not in equilibrium with its receptors (Section 2.3.2).

2.3.1. Complete competitive antagonist-receptor equilibrium

If K_B is unknown, then K_A may be estimated by utilizing data obtained from Fig. 2. According to Eq. (10), K_A may be estimated graphically by plotting the $[A]_{1/2}$ values of the sub-maximal curves against $\phi[A]_{1/2}$. The plot of $[A]_{1/2}$ on $\phi[A]_{1/2}$ gave a straight line and K_A was estimated from its intercept with the ordinate (inset: Fig. 2).

If K_B is known, then K_A may be estimated from the slope of the previous straight line, or by utilizing curve heights (h). The heights (h) (Table 1), which may be measured in arbitrary units, were measured as fractions of the maximal effect ($E = 1.0$) (Fig. 2) and were plotted against ϕh yielding a straight line as is predicted by the theory (inset: Fig. 3). The K_A value was calculated by utilizing the K_B value and the slope of the straight line.

The dissociation constant K_A can also be estimated directly from a single agonist-antagonist concentration-effect curve (Fig. 1) by converting Eq. (8) into the following form:

$$K_A = \frac{[A]_{1/2}}{1 - \phi[A]_{1/2}/K_B} \qquad (14)$$

The concentration ratio ϕ for the concentration-effect curve is a known constant and $[A]_{1/2}$ was obtained directly

Table 1
Heights of concentration-effect curves of agonist-competitive antagonist combinations for different values of ϕ (from Fig. 2 and Fig. 3)

Curve no.	Curve height	ϕ [a]	ϕh
h_1	0.3401357	3.2	1.088434
h_2	0.54945	1.6	0.87912
h_3	0.7936514	0.8	0.634921

[a] $\phi = [B]/[A]$.

from the agonist-antagonist curve. It also follows from Eq. (14) and the relationship $[B]_{1/2} = \phi[A]_{1/2}$, that:

$$K_A = \frac{[A]_{1/2}}{1 - [B]_{1/2}/K_B} \quad (15)$$

If pure competitive antagonism is at stake, then it follows from Eq. (14) that $1 > \phi[A]_{1/2}/K_b$ and from Eq. (15) that $1 > [B]_{1/2}/K_B$. Unfortunately, the relationship $1 > \phi[A]_{1/2}/K_B$ (or $1 > [B]_{1/2}/K$) may restrict this method in its application since it imply that only fairly accurate experimental results would be suitable for the analysis. Values of $\phi[A]_{1/2}/K_B > 1$ will produce negative values for K_A which are meaningless. Therefore, in an analyses where the actual value of $\phi[A]_{1/2}/K_B$ is close to one (i.e. $\phi[A]_{1/2}/K_B \approx 1$), negative values would certainly be obtained in a number of the experiments due to experimental errors. The ideal situation would of course prevail when $\phi[A]_{1/2}/K_B$ is much smaller than one (i.e. $\phi[A]_{1/2}/K_B \ll 1$). In this last instance one would expect a minimum number of negative results.

2.3.2. *Incomplete competitive antagonist-receptor equilibrium*

The classical method of K_B estimation, as well as the above mentioned methods, are based on the assumption that the concentration of a competitive antagonist at the receptors (i.e. in the biophase) is equal to, or at least linearly related to that in the surrounding medium (fluid in organ bath). If this assumption of concentration equality is not met then incorrect dissociation constants, K_B, may be obtained (Kenakin, 1984). It is important, therefore, that sufficient equilibrium time must be allowed to produce a thermodynamic equilibrium between the antagonist and its receptors. When a concentration-effect curve is determined with an agonist-competitive antagonist combination, it may be possible that the prerequisite of sufficient time to attain equilibrium may not be met for certain antagonists. One approach is to circumvent this difficulty by employing antagonists which will equilibrate with the tissue within the time space of the particular experiment, i.e. the competitive antagonist used in the agonist-antagonist combination should reach equilibrium in the same, or shorter, time span than does the agonist. If such an antagonist is not available, then this problem may be overcome by employing Eq. (16), which is obtained by combining Eq. (15) and the following form of Eq. (13):

$$K_B = \frac{[B]_{1/2}}{[A]_{1/2}/[A]_1 - 1}$$

$$K_A = \frac{[A]_{1/2}}{2 - [A]_{1/2}/[A]_1} \quad (16)$$

$[A]_1$ represents a concentration of the agonist A (in absence of any antagonist) which gives the same quantitative effect as $[A]_{1/2}$. From Eq. (16) K_A can be estimated directly, even if the actual values of K_B and [B], or $[B]_{1/2}$, are unknown. Since $[B]_{1/2}$ in the relationship $[B]_{1/2}/K_B$, in Eq. (15), relates to the actual concentration of B interacting with the receptors in the biophase, the value of $[A]_{1/2}$ (evaluated experimentally) is actually determined by $[B]_{1/2}$ while K_B is a constant unrelated to concentration. Therefore, the correct value of K_A may be estimated from Eq. (16) even though equilibrium was not accomplished for the antagonist-receptor interaction. It boils down to the fact that K_A may be estimated even though the values of K_B and [B], i.e. $[B]/[A] = \phi$, are unknown.

The relationship $[A]_{1/2}/[A]_1 < 2$ may restrict this method in its application since values of $[A]_{1/2}/[A]_1 > 2$ will produce negative K_A values. Therefore, only fairly accurate experimental results would be suitable for analysis.

Eq. (16) can be modified to give the following linear equation:

$$[A]_{1/2} = -K_A \frac{[A]_{1/2}}{[A]_1} + 2K_A \quad (17)$$

A straight line which intercepts the ordinate at $[A]_{1/2} = 2K_A$ was obtained by plotting $[A]_{1/2}$ against $[A]_{1/2}/[A]$ (inset: Fig. 1). The K_A value may also be estimated from the slope ($= -K_A$) of the straight line.

2.4. *Estimation of agonist affinity when stimulus and effect curves do not coincide*

If a nonlinear stimulus-effect relationship is present in a system due to receptor reserve for half-maximal effect, then, clearly, the concentration-stimulus curves and concentration-effect curves of the interacting agonist will not coincide (Fig. 4). It is of course possible that a receptor reserve may exist for half-maximal effect as well as for maximal effect in a particular agonist-effector system. If the latter conditions prevail in the agonist-effector system, then it would be impossible for the concentration-stimulus curve and concentration-effect curve of the agonist to coincide (Fig. 4).

When applying the model to agonist-effector systems where one of the above mentioned nonlinear relationships prevail, the model automatically operates as if the stimulus and effect curves of the agonist coincide to a lesser or larger extend. Therefore, the experimentally determined affinity values of agonists A_1 and A_2, namely $K_{A_1}(e)$ and $K_{A_2}(e)$, will most certainly differ from the actual affinity values of A_1 and A_2, namely K_{A_1} and K_{A_2} (see Fig. 4). It is important to note that the model takes reserve receptors for maximal effect into account when $K_{A_2}(e)$ is estimated. It is, however, clear that deviation from the supposition in Section 2.3, namely that concentration-stimulus curves and concentration-effect curves should coincide to some extend, introduces an error into affinity measurements the magnitude of which depends upon the nature of the function relating stimulus and effect.

This problem may be solved by utilizing relative affinity values rather than absolute affinity values. If the apparent affinity (estimated by the new method) of two or more agonists is compared directly in the same tissue and on the same receptor type, then tissue factors will be cancelled and their relative affinity will depend only upon the actual drug-receptor affinity since the receptor number and efficiency of stimulus transduction is supposed to be the same for the agonists in question. If the latter supposition holds, then: $K_{A_1}/K_{A_2} = K_{A_1}(e)/K_{A_2}(e)$. Therefore, if the $K_{A_1}(e)$ value of agonist A_1 is arbitrary set equal to $Rel - K_{A_1}$, then the affinity of agonist A_2 relative to A_1, namely $Rel - K_{A_2}$, can be calculated according to: $Rel - K_{A_1} \times K_{A_2}(e)/K_{A_1}(e)$.

2.5. Estimating K_A values by utilizing literature data

The ultimate test for the usefulness of the new approach depends on the following questions: (1) can reliable pharmacological data be obtained from which the K_A values could be estimated; and (2) would this new approach lend itself for determining dissociation constants by utilizing published data? Regarding the proposed methods the answers to both these questions are an unequivocally 'yes'. By analyzing a set of concentration-effect curves of an agonist A determined in the absence and presence of different fixed concentrations of a competitive antagonist B, and utilizing Eq. (12), one can easily estimate the apparent affinity (K_A) of the agonist for its receptors. The heights (h) of agonist-antagonist concentration-effect curves may be deduced from such sets of agonist concentration-effect curves which are found abundantly in the pharmacological literature. The procedure of deducing agonist-antagonist concentration-effect curves from them is illustrated in Fig. 3. The set of agonist concentration-effect curves obtained in the absence and presence of increasing antagonist concentrations, shown in Fig. 3, was calculated by means of Eq. (3). Theoretical concentration-stimulus curves for agonist A were calculated for constant values of K_A, K_B and S_m ($K_A = K_B = 1$ μM, $S_m = 1$) while the value of [B] was increased from zero to 300 μM. The theoretical concentration-effect curves (continues lines), simulating experimental concentration-effect curves, were constructed according to the assumption that the maximal effect ($E = 1$) on the effector is obtained at a stimulus value of $S_{AB} = 0.7$. From this set of concentration-effect curves one can easily determine the height (h), that should have been reached by the sub-maximal concentration-effect curves mediated by fixed agonist-antagonist combinations (for constant ϕ). The positions of constant values for ϕ, located on the different concentration-effect curves of the agonist determined in the presence various [B], where calculated by means of Eq. (4). These positions were marked on the different concentration-effect curves of agonist A, yielding an agonist-antagonist concentration-effect curve which is associated with a fixed [B]/[A] ($= \phi$) ratio. Concentration-effect curves which have been deduced by this approach are shown in Fig. 3 as broken lines. The heights (h), which may be measured in arbitrary units, were measured as fractions of the maximal effect ($E = 1.0$) (Table 1), and were plotted against ϕh yielding a straight line as predicted by the theory. The K_A value was estimated from the slope of the straight line (inset: Fig. 3).

Table 2
Heights of concentration-effect curves of carbachol-tripitramine combinations for different values of ϕ

h [a]	ϕ [b] $\times 10^{-2}$	$\phi h \times 10^{-2}$
0.2913	7.7843	2.2673
0.3398	6.2067	2.1091
0.3883	5.0059	1.9441
0.4369	4.1329	1.8056
0.4854	3.4129	1.6567
0.5340	2.8396	1.5163
0.5825	2.3723	1.3819
0.6311	1.9441	1.2268
0.6796	1.6163	1.0984
0.7282	1.3166	0.9587

The carbachol-tripitramine curves are deduced from carbachol curves determined in the absence and presence of different fixed tripitramine concentrations on M_3 receptors of guinea-pig ileum (Chiarini et al., 1995). [a] h = fraction of maximal effect (sub-maximal curve height)/(maximal curve height). [b] ϕ = [tripitramine]/[carbachol].

In the next phase the above mentioned procedure was put into practice by estimating apparent K_A from literature data. For this purpose a set of agonist concentration-effect curves, determined in the absence and presence of increasing concentrations of a competitive antagonist, was analyzed. The set of agonist concentration-effect curves was determined for carbachol in the absence and presence of increasing tripitramine concentrations (5, 10, 50 and 100 μM) on muscarinic M_3 receptors in guinea-pig ileum (Chiarini et al., 1995). The heights (h) of concentration-effect curves deduced for fixed carbachol/tripitramine concentration ratios were obtained from this set of experimental concentration-effect curves and are shown in Table 2. In deducing the series of carbachol-tripitramine concentration-effect curves, only maximal effects were obtained in the presence of the greatest tripitramine concentrations ([tripitramine] = 50 and 100 μM). Therefore, the ϕ values for several carbachol-tripitramine curves could readily be estimated at different heights (effects) of choice (Table 2).

A plots of h against ϕh resulted in the expected straight line from which the apparent K_A value of the carbachol-M_3 receptor complex was estimated (Fig. 5). The data points were fitted to a straight line by the method of least squares.

3. Results

Figs. 1 and 2 show theoretical results, which simulate experimental results, of a full agonist and agonist-

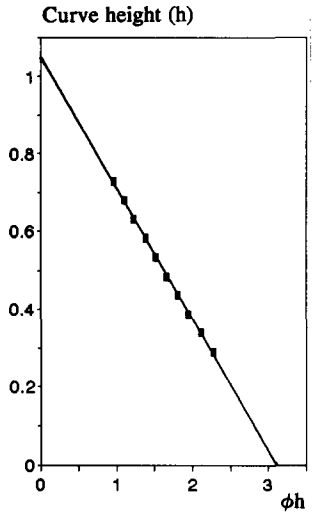

Fig. 5. A plot of relative curve heights (h) against ϕh (Table 2) for carbachol-tripitramine combinations on M_3 receptors of guinea-pig ileum (Chiarini et al., 1995). The data points were fitted to a straight line by the method of least squares. Slope = -0.336 ± 0.003; ordinate intercept: 1.046 ± 0.004; Correlation coefficient, r: 0.999.

antagonist combination(s) on effector(s), when a nonlinear relationship (spare receptors) prevails for the stimulus-effect relationship of the agonist. The simulated concentration-effect curves in Figs. 1 and 2 show the influence of a competitive antagonist B on an agonist A for different values of $\phi =$ [B]/[A]. When $\phi = 0$, i.e. [B] = 0, a theoretical concentration-effect curve of a full agonist A with $EC_{50} = 0.53847$ μM was obtained. The combination of agonist A and competitive antagonist B in fixed concentration ratios ($\phi = 0.8$, 1.6 and 3.2 in Fig. 2) simulated partial agonists, i.e. relative to the full agonist A these agonist-antagonist combinations mediated sub-maximal effects on the effector.

Fig. 1 was used to estimate K_A values when equilibrium has not been attained for the competitive antagonist-receptor interaction. In this case Eq. (16) and data obtained from Fig. 1 were utilized to calculate K_A directly. By plotting $[A]_{1/2}$ against $[A]_{1/2}/[A]_1$, Eq. (17), a straight line was obtained and the affinity ($K_A = 1$ μM) of agonist A for its receptors was estimated from its slope and/or the intercept with the ordinate (inset: Fig. 1). The straight line is described by the following equation:

$$[A]_{1/2} = -1.0[A]_{1/2}/[A]_1 + 2.$$

The values of $[A]_{1/2}$ and $[A]_1$, for agonist-antagonist concentration-effect curves of different ϕ values, were obtained from the theoretical concentration-effect curves shown in Fig. 2.

The simulated concentration-effect curves in Fig. 2 represent the effects determined in separate experiments of agonist-antagonist combinations and for different concentration ratios (ϕ) of agonist-antagonist combinations. The values $[A]_{1/2}$ and $\phi[A]_{1/2}$ were obtained form the theoretical concentration-effect curves in Fig. 2. A plot of $[A]_{1/2}$ against $\phi[A]_{1/2}$ yielded a straight line as was predicted by Eq. (10) (inset: Fig. 2). The straight line is described by the following equation:

$$[A]_{1/2} = -1.0\phi[A]_{1/2} + 1.$$

From the intercept with the ordinate and/or slope of the straight line it was estimated that $K_A = 1.0$ μM.

The heights (h) of the sub-maximal curves in Fig. 2 (i.e. when $\phi = 0.8$, 1.6 and 3.2) were also used to estimate K_A by employing Eq. (12). By plotting h against ϕh a straight line was obtained (inset: Fig. 3 and Table 1), which is described by the following linear equation:

$$h = -1.0\phi h + 1.4286$$

It followed from the slope of this straight line that $K_A = 1.0$ μM if $K_B = 1$ μM. From the intercept with the ordinate it followed that $h = S_m = 1.4286$, where S_m represents maximal the height of the stimulus curve of agonist A, relative to the maximal height of the effect curve ($E = 1.0$) of A. This means, of course, that the maximal height of the concentration-stimulus curve (S_m) is 1.4286 times greater than the maximal height of the concentration-effect curve, i.e. if $E = 1.0$ then $S_m = 1.4286$. It follows that if S_m is arbitrary set equal to unity, i.e. $S_m = 1$, then maximal effect ($E = 1$) would correspond to $S_{AB} = 1/1.4286 = 0.7$ (see Fig. 2).

Fig. 3 shows the theoretical concentration-effect curves and concentration-stimulus curves of a full agonist A in the absence and presence of increasing concentrations of a competitive antagonist B calculated according to Eq. (3). The continues curves represent simulated experimental concentration-effect curves and dotted curves represent concentration-stimulus curves when spare receptors are present in the system. The maximal effect ($E = 1.0$) of the full agonist A was obtained at a stimulus value of $S_{AB} =$ 0.7 and the EC_{50} of the agonist A was obtained at 0.53847 μM. The broken lines in Fig. 3 represent agonist-antagonist curves (for which $\phi = 0.8$, 1.6 and 3.2) deduced from data obtained from the simulated concentration-effect curves of A in the presence of various [B]. The heights (h), as fractions of the maximal effect, of the deduced agonist-antagonist concentration-effect curves were obtained from these curves (broken lines) and are shown in Table 1. By plotting h against ϕh a straight line was obtained (inset: Fig. 3), which is described by the following equation:

$$h = -1.0\phi h + 1.4286$$

Fig. 5 shows a plot of curve heights (h) against ϕh (Table 2) for experimental carbachol-tripitramine combinations. As predicted by receptor theory, i.e. Eq. (12), plots of the heights (h) against the product ϕh for carbachol-tripitramine curves resulted in a straight line which is described the following linear Eq. (Fig. 5):

$$h = -0.336\phi h + 1.046$$

From the slope of this linear relationship was estimated that apparent K_A for the carbachol-muscarinic (M_3) recep-

tor complex is $1.06 \times 10^{-1} \pm 0.013$ μM. In calculating the K_A a K_B value (tripitramine) of $3.16 \times 10^{-1} \pm 0.036$ μM was used (Chiarini et al., 1995). The $EC_{50} = 8.91 \times 10^{-2}$ μM of carbachol was determined from the literature data. From the ordinate intercept of the linear regression was estimated the maximal height of the carbachol stimulus curve ($h = S_m = 1.046 \pm 0.004$), relative to the maximal height of the effect curve.

4. Discussion

The theory of the proposed methods for estimation of agonist affinities is based on the model for competitive antagonism which has been very successful in relating drug parameters. The elegance of these new methods is attributed firstly, to the fact that it is unnecessary to make additional assumptions or approximations, secondly, to its ability to employ any known competitive antagonist to attain the desired pharmacological data, and thirdly, to the possibility that a variety of pharmacological methods may be employed, i.e. one may utilize concentration-effect curves of fixed agonist-antagonist combinations, or agonistic concentration-effect curves determined in the presence of increasing concentrations of a competitive antagonist. The choice of competitive antagonist to be used in experiments is solely based on the properties of the antagonist in question. Since the Schild regression is known as a powerful tool in establishing simple competitive antagonism it can be verified beforehand, by applying the Schild regression, that a drug is indeed a competitive antagonist. By selecting, for instance, an antagonist that is easily removed from the receptor compartment when the tissue is washed with fresh bathing medium, it can be ensured that various curves may be determined on a single isolated organ. Therefore, one can ensure a convenient and reliable test system by choosing a competitive antagonist which possesses properties optimally suited for particular K_A estimations.

A feature of some the methods reported in this paper is the necessity that the dissociation constant of the antagonist-receptor complex (K_B) should be known. It is therefore of paramount importance that reliable K_B values should be available for estimating K_A values. Fortunately, in this regard, it is generally accepted that measurements of antagonist affinities can be achieved with considerable accuracy (Van den Brink, 1977; Kenakin, 1987b, Kenakin, 1987c).

Distinct advantages of these new methods are as follows: (1) the values of efficacy (e) and/or intrinsic efficacy need not be known for estimation of K_A; (2) possible reserve receptors for maximal effect is taken into account and therefore reflects in the estimated K_A values; and (3) these new methods give rise to a number of different procedures that may be utilized to estimate K_A values, for example, K_A may be estimated indirectly by graphical means (see Figs. 1–4) or directly by employing Eqs. (14) and (15). It was pointed out, however, that the employment of the latter two equations require the use of fairly accurate pharmacological data. Although this may restrict the use of Eqs. (14) and (15) it should certainly not render them useless.

Graphical estimation of K_A values afford the opportunity to employ a variety of experimental techniques. Firstly, the concentration which refers to the concentration at which half maximal curve height (h/2) is obtained, namely $[A]_{1/2}$, may be used in graphical estimations of K_A values (see Eq. (10) and Fig. 2). Secondly, the K_A value may be estimated graphically (inset: Fig. 3) by employing Eq. (12) and utilizing the heights (h) of the set of agonist-antagonist concentration-effect curves shown in Fig. 2. In the application of these methods it was assumed that the concentration-stimulus curves and concentration-effect curves will coincide when a sub-maximal effect is obtained with agonist-antagonist combinations. It was also assumed in all these methods that the competitive antagonist is in equilibrium with its receptors. If the concentration-effect curves are determined with agonist-antagonist combinations, then one can employ only antagonists that reach equilibrium within the same time space allowed for the agonists to reach equilibrium. It is of course further assumed in this method, as in all other methods currently employed, that the responses are due to concentrations of agonists at the receptor which are equal to those in the organ bath. This latter point is of crucial importance to all pharmacological experiments designed for K_A estimation and it has been emphasized repeatedly in the literature (Schild, 1957; Van Rossum, 1966; Kenakin, 1984).

If it is suspected that the competitive antagonist concentration in the biophase (region where the receptors are located) differs from the antagonist concentration in the bath fluid, i.e. if equilibrium is not accomplished for the antagonist-receptor interaction, then K_A values may be estimated directly by employing Eq. (16) or graphically by employing Eq. (17). These latter equations provide a means to estimate K_A when K_B and [B], i.e. $[B]/[A] = \phi$, are unknown.

When estimates of spare receptors are made, pharmacologists usually rely solely on differences between the K_A and EC_{50} values. Erroneous conclusions could be drawn if the difference between the K_A and EC_{50} values is used as the sole premise to establish the existence in a tissue of spare receptors for a particular drug. However, Eq. (12) affords the opportunity to estimate the maximal height of a concentration-stimulus curve ($h = S_m$) which may indicate whether or not spare receptors are present in the system. Reserve receptors for maximal effect will be present if $S_m > 1.0$. This aspect is discussed thoroughly in a following paper.

It was shown further that Eq. (12) also provides a facile method for estimating K_A values by utilizing sets of agonist concentration-effect curves determined in the ab-

sence and presence of increasing competitive antagonist concentrations (Fig. 3). Pharmacological data obtained for agonists in the presence of different fixed competitive antagonist concentrations are usually obtained under absolute controlled conditions which would certainly contribute to the estimation of reliable pharmacological parameters. By using this method one can choose the optimum contact time to ensure that the competitive antagonist is in equilibrium with its receptors before determining the agonistic concentration-effect curves. From such a set of agonist curves the heights (h) of deduced concentration-effect curves for agonist-antagonist combinations may be calculated for different values of $\phi = [B]/[A]$. The practical usefulness of the latter procedure was put to test by analyzing a set of agonist (carbachol) concentration-effect curves determined in the absence and presence of increasing concentrations of a competitive antagonist (tripitramine). Data from this set of curves was utilized to deduce concentration-effect curves for carbachol-tripitramine combinations at different values of ϕ. The heights (h) of these curves (Table 2) were used for the graphical estimation of the K_A value for the carbachol-M_3 receptor complex (Fig. 5). It was found for carbachol that the K_A value is of about the same order than the EC_{50} value. Since no information about the exact location of the concentration-stimulus curves are known, this experimentally determined K_A value should not be regarded as an absolute quantity and it should thus actually be treated as an apparent K_A value. The finding that the maximal height of the concentration-stimulus curve of carbachol equals the height of its concentration-effect curve, suggests the absence of reserve receptors for the maximal effect.

Unfortunately, the actual K_A values of agonist-antagonist complexes can only be obtained from concentration-stimulus curves (which cannot be determined experimentally), or from concentration-effect curves which actually coincide with the concentration-stimulus curves when sub-maximal effects occurs. The possibility that the latter assumption will hold in most systems is probably rather slim. The most likely possibility is that concentration-effect curves and their parent concentration-stimulus curves would have totally different locations above the concentration axis, and therefore, as is illustrated in Fig. 4 they would not coincide at all. Because of the latter possibility it was suggested that relative affinities rather than isolated K_A values should be estimated. In the utilization of relative K_A values it is expected that postreceptor events should be cancelled, and thus play no role in the estimation of relative K_A values. Therefore, affinity values chosen relative to the apparent K_A of a reference compound should yield a highly reliable affinity scale. It is clear that a scale of relative affinities will give exactly the same information as is obtainable from the mutual comparison of different isolated K_A values, because, in practical terms an isolated K_A is actually meaningless and the real usefulness of K_A values would only emerge when various K_A values of different agonists are mutually compared. In any case, suppose the relative affinities of a series of agonists are known, then the correct affinity values (K_A) of all the agonists in the series can easily be calculated if the exact affinity value (K_A) of only a single agonist in the series is known.

The possibility of functional interaction within the effector may influence the height (h) of the curves, and thus also the value of $[A]_{1/2}$. For instance, α-adrenoceptors in the rat vas deferens mediate contraction effects while β_2-adrenoceptors mediate relaxing effects (Vohra, 1979; Lotti et al., 1980). Fortunately, this possible adverse influence of functional interaction may be eliminated by blockade of the unwanted drug-receptor interaction (effects) with an appropriate competitive antagonist (Szabadi, 1977).

In conclusion, it has been demonstrated that the methods for estimating the relative dissociation constants and dissociation constants for agonist-receptor complexes reported in this paper may be superior to methods currently employed. The estimation of the relative agonist affinity by utilizing these methods takes reserve receptors into account, and therefore, it should yield true relative affinities which furnish reliable information to the medicinal chemist so that meaningful structure-activity relationships can be made for the design of better therapeutic drugs. Furthermore, regarding the ease of practical application, this new approach provides a straightforward method for estimating dissociation constants and relative dissociation constants. The analyses discussed in this paper show that agonist concentration-effect curves determined with fixed ratios of agonist-antagonist combinations may unlock dependable information, that as yet was not readily obtainable from concentration-effect curves.

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