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A TEST OF THE NULL EQUATION FOR FUNCTIONAL ANTAGONISM

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1 The quantitative model for functional antagonism and synergism has been tested by studying its ability to fit data obtained from the functional antagonism of (−)-isoprenaline by muscarinic agonists on guinea-pig isolated atria.

2 The general form of the null equation has been shown to fit the experimental curves satisfactorily.

3 Functional interaction between (−)-isoprenaline and muscarinic agonists on atria has been shown to be type I although there does seem to be a discrepancy between values of the functional affinity constants, $K_{A1}{}^F$ and $K_{A2}{}^F$, estimated in two different ways.

4 The affinity constants, K_A, of the muscarinic agonists for their receptors have been estimated by use of the selective irreversible antagonist propylbenzilylcholine mustard. The discrepancy between $K_A{}^F$ (i.e. both $K_{A1}{}^F$ and $K_{A2}{}^F$) and K_A is small for pentyltrimethylammonium which is an agonist of low intrinsic efficacy. By contrast the discrepancy between $K_A{}^F$ and K_A is much greater for methylfurmethide and oxotremorine both of which have much higher intrinsic efficacies. These results are as predicted by the model.

5 It is suggested that the discrepancy between $K_{A1}{}^F$ and $K_{A2}{}^F$ may be due to the limited ability of the equation

$$1/S_{\beta} = a_1 + b_1/S_{\alpha}$$

to describe quantitatively the relation between sequential stimuli. However, it is concluded that this complication need not interfere with the use of the model to study mechanisms and possible sites of functional interaction.

Introduction

In the preceding paper (Mackay, 1981) null equations were derived for functional antagonism and synergism on the basis of several assumptions. Although the null equation for one particular type of functional interaction, designated type I, was shown to be in good qualitative agreement with the results of Van den Brink (1973a, b) more detailed quantitative tests seem desirable. The results of such tests are described in this paper using data obtained from experiments on the interaction of (−)-isoprenaline with muscarinic agonists on guinea-pig isolated atria.

Theory

According the the model discussed in the previous paper, if two state-concentration curves for an agonist A are measured on a cell or tissue, each curve being determined in the presence of a different concentration of a functional interactant, then the concentrations $[A]_1$ and $[A]_2$ of the agonist required to produce equivalent states of the tissue should fit the general null equation

$$[A]_2/[A]_1 = \alpha + \beta[A]_2 + \gamma/[A]_1 \qquad 1$$

where α, β and γ are adjustable constants for each pair of state-concentration curves. The values of α, β and γ and their interdependence, if any, may provide information about the possible mechanism of interaction. The theoretical criteria for classifying the various types of functional interaction were set out in Table 1 of the previous paper (Mackay, 1981). According to the model the affinity constant of an agonist for its receptors can only be estimated dependably from such data if the interaction is type IIA. If the interaction is type I then the model predicts that α, β and γ are not independent since $4\beta\gamma = (\alpha - 1)^2$. In these circumstances it should be possible to estimate two values for the functional affinity constant, namely

$$K_{A1}{}^F = (\alpha - 1)/2\gamma \text{ and } K_{A2}{}^F = 2\beta/(\alpha - 1) \qquad 2a,b$$

which should be equal and related to the true affinity constant K_A through the equation

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$$K_A^F = K_A[a_I f_A R_I / b_I + 1] \quad 3$$

where a_I and b_I are chain constants and f_A and R_I are respectively the intrinsic efficacy of agonist A and the total concentration of receptors on which it acts.

If on the other hand two agonists A and B act on the same receptors in the same piece of tissue maintained in a *constant initial state* then the null equation which relates their two concentration-response curves (Mackay, 1966) is

$$1/[A] = I_{AB} + \psi_{AB}/[B] \quad 4a$$

or

$$[B]/[A] = \psi_{AB} + I_{AB}[B] \quad 4b$$

where I_{AB} and ψ_{AB} are constants such that

$$I_{AB} = K_A[f_A/f_B - 1] \quad 5a$$

and

$$\psi_{AB} = f_A K_A / f_B K_B \quad 5b$$

If A and B are full agonists then I_{AB} is zero and ψ_{AB} becomes the relative potency of agonists A and B. If these agonists interact functionally with another substance by a type I mechanism then from equations 3 and 5b it can be deduced that

$$\log[K_A^F - K_A] = \psi_{AB} + \log[K_B^F - K_B] \quad 6$$

provided that K_A^F and K_B^F are measured on the same piece of tissue. If agonist B is kept constant, as a reference compound, while A is varied then $\log[K_A^F - K_A]$ should be linearly related to ψ_{AB}, with a slope of unity. Equation 6 would also be expected to hold for average values of K_A^F and K_B^F estimated on a sufficiently large number of samples of the tissue. Since all of the quantities in equation 6 can be estimated experimentally it provides an indirect test of equation 3.

Methods

Isolated left atria were obtained from guinea-pigs (350 to 650 g) of either sex which had been treated with reserpine (2.5 mg/kg i.p.) 20 h before the experiment. The atria were set up at 35°C in Krebs solution (Krebs & Henseleit, 1932) with the addition of glucose (11 mM), ascorbic acid (1.1 mM) and phentolamine (10 μM). Metanephrine (50 μM) was also present during the determination of cumulative curves, being added directly to the organ bath approximately 30 min before the estimation of each curve. Although these concentrations of phentolamine and metanephrine hardly affect the sensitivity of the atrium to isoprenaline, they do change the sensitivity of the guinea-pig isolated trachea (O'Donnell & Wanstall, 1977). It was therefore decided to use these concentrations of phentolamine and metanephrine routinely so that the experiments on atria would be carried out under the same experimental conditions as other experiments at present being done on trachea.

The atria were stimulated at 3 Hz using rectangular pulses of 1 ms duration and 5 V amplitude (supramaximal). The resting tension was 0.5 g and contractions were recorded isometrically. All force-concentration curves for the muscarinic agonists were estimated cumulatively in the presence of known concentrations of (−)-isoprenaline sufficient to produce positive inotropic effects in the range 40% to 100% of maximal. Each dose of isoprenaline was added 5 min before beginning the estimation of each cumulative curve for the muscarinic agonists. The tissue was washed by overflow and allowed to recover for 30 min between the determination of successive cumulative curves. In those experiments in which propylbenzilylcholine mustard (PrBCM) was used to reduce the number of available muscarinic receptors in the tissue the selective irreversible antagonist (0.02 μM to 0.04 μM) was left in contact with the tissue for 3 to 6 min before being washed out. The degree of muscarinic blockade produced by PrBCM was not entirely predictable and repeated treatments were sometimes required to produce appropriate displacement of the force-concentration curves. Whenever possible, one force-concentration curve was repeated intermittently during an experiment to detect any spontaneous change in tissue sensitivity.

Drugs

Reserpine, (−)-isoprenaline bitartrate, oxotremorine sesquifumarate, and DL-metanephrine hydrochloride were all obtained from Sigma, and atropine sulphate from BDH. We are especially grateful to Dr J.M. Young for the gift of a sample of propylbenzilycholine mustard, to Ciba for phentolamine mesylate, and to Mr R. Blakeborough and Dr J. Wheeler for preparing the samples of methylfurmethide and pentyltrimethylammonium bromide respectively.

Comparison of state-concentration curves and analysis of results

Only those curves have been analysed which were obtained during periods when the sensitivity of the tissues seemed to be reasonably constant. The force of contraction of the atrium was taken as a measure of its state. Smooth curves were drawn by eye through plots of force versus the log of the concentration of muscarinic agonist. The concentrations of agonist which produced equivalent states were read from these smooth curves at force levels spread fairly evenly through the force range common to both

Table 1 Distribution of values of α, β and γ estimated for muscarinic agonists acting against (−)-isoprenaline on guinea-pig isolated atria

Agonist	*Number of times the parameter exceeds zero*			*Number of individual values which exceed 2 standard errors*		
	(α–1)	β	γ	(α–1)	β	γ
Pentyl-TMA	8	8	8	8	5	8
Methylfurmethide	9	6	9	8	4	9
Oxotremorine	3	1	3	1	0	3
Totals	20	15	20	17	9	20

The number of results for pentyltrimethylammonium (pentyl-TMA), methylfurmethide and oxotremorine were 8, 9 and 3 respectively making a total of 20 results.

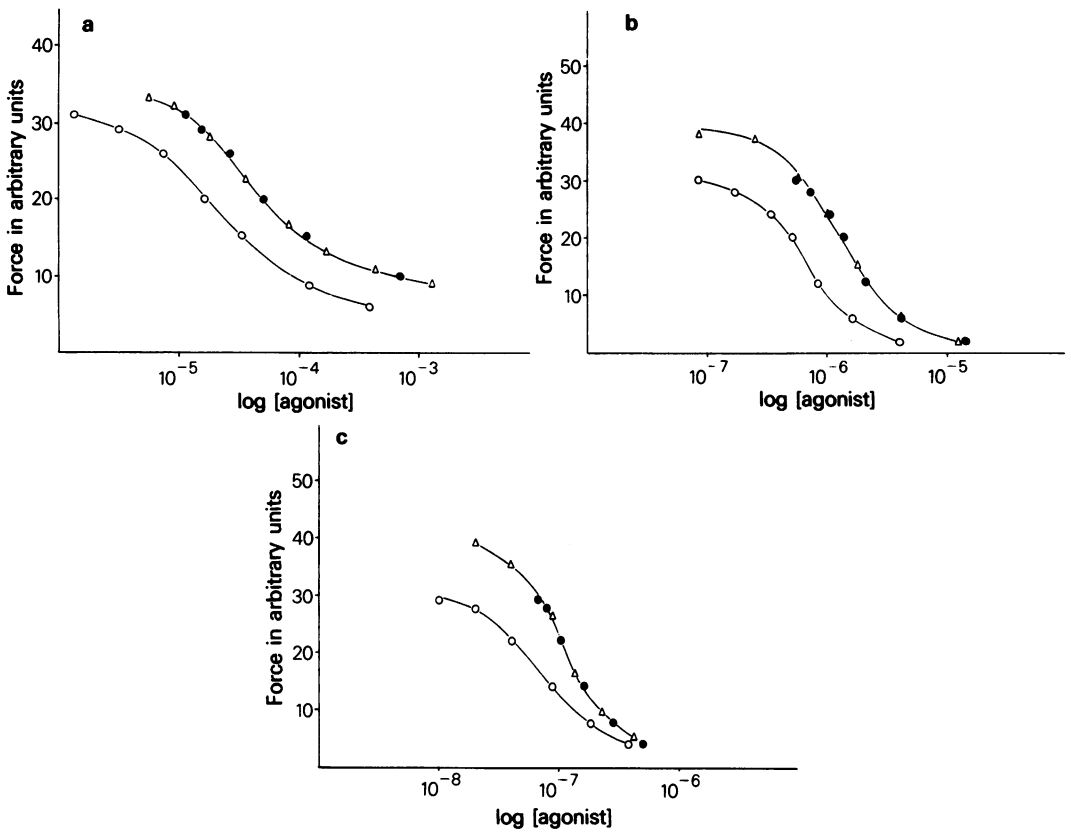

Figure 1 Illustrations of the ability of the general null equation to fit experimental data which in each case consists of force-log concentration curves for the appropriate muscarinic agonist obtained in the presence of two concentrations of (−)-isoprenaline: (○) and (△) indicate actual experimental points; (●) indicates a theoretical value calculated using values of α, β and γ obtained by fitting the null equation to the experimental data. In cases (b) and (c) the values of β do not differ significantly from zero. (a) Pentyltrimethylammonium: $\alpha = 2.117$, $\beta = 8.593 \times 10^3$ and $\gamma = 8.437 \times 10^{-6}$. (b) Methyl furmethide: $\alpha = 1.82$, $\beta = 1.11 \times 10^5$ and $\gamma = 3.91 \times 10^{-7}$. (c) Oxotremorine: $\alpha = 1.228$, $\beta = -9.43 \times 10^4$ and $\gamma = 5.478 \times 10^{-8}$.

curves. The number of such force levels chosen was equal to the smaller number of total experimental points in either of the two curves (see Figure 1). A computer programme was used to fit equation 1 to such data read from cumulative curves obtained for any one muscarinic agonist in the presence of different concentrations of (−)-isoprenaline. Although the programme provided estimates of α, β and γ together with their standard errors the latter are likely to be underestimated since they are based on 'smoothed' data. Nevertheless even such approximate standard errors give some guide to the relative dependability of individual estimates of α, β and γ. Estimates of $K_{A1}{}^F$ and $K_{A2}{}^F$ (see equations 2a, b) and their approximate standard errors were obtained from the values and standard errors of α, β and γ.

The affinity constant of atropine as an antagonist of the various muscarinic agonists, the values of ψ_{AB} (or relative potencies) of the muscarinic agonists and estimates of their affinity constants for their receptors (obtained by using PrBCM) were all determined in an analogous way by comparing the appropriate force-log concentration curves obtained on a single piece of tissue in the presence of the same concentration of (−)-isoprenaline. The same computer programme was used to analyse such data since the null equations in these circumstances (Mackay, 1966) are merely special forms of equation 1 with γ equal to zero for the comparison of agonists and both β and γ equal to zero for competitive antagonism.

Results

The affinity constants estimated for atropine acting as an antagonist of the three agonists pentyltrimethylammonium (pentyl-TMA), methylfurmethide and oxotremorine were 0.328 ± 0.068, 0.317 ± 0.071 and 0.254 ± 0.071 respectively, each mean and standard error being based on 4 results and expressed in nM^{-1}.

Experiments on the functional antagonism of (−)-isoprenaline by these three muscarinic agonists on atria not pretreated with PrBCM yielded values of α, β and γ the distributions of which are summarized in Table 1. In order to illustrate better the ability of equation 1 to fit such data values of α, β and γ, estimated from one typical experiment involving each muscarinic agonist, have been used to calculate theoretical values of $[A]_2$ corresponding to each experimental value of $[A]_1$ using the equation

$$[A]_2 = (\alpha[A]_1 + \gamma)/(1 - \beta[A]_1)$$

which is an alternative form of equation 1. The results of such calculations are shown in Figures 1a, 1b and 1c.

Equations 2a and 2b were used to convert values of α, β and γ into estimates of $K_{A1}{}^F$ and $K_{A2}{}^F$. The number of experiments in which $K_{A1}{}^F$ exceeded $K_{A2}{}^F$ is recorded for each agonist in Table 2, excluding entirely those experiments in which either $K_{A1}{}^F$ or $K_{A2}{}^F$ was negative or was less than its own standard error. The mean values of $K_{A1}{}^F$ and $K_{A2}{}^F$, estimated from these same paired values, are also summarised in Table 2.

Estimates of $K_{A1}{}^F$ and $K_{A2}{}^F$ are again presented in Table 3, this time for experiments on atria treated with PrBCM as well as on untreated atria. In preparing this table, all individual values were included unless they were negative or were less than their own standard error. Values of the affinity constants of the three muscarinic agonists for their receptors, estimated by use of PrBCM, are also included in Table 3, together with estimates of ψ_{AB} for each agonist relative to pentyl-TMA. The intrinsic efficacies of methylfurmethide and oxotremorine relative to pentyl-TMA have been estimated from the appropriate values of ψ_{AB} and K_A and are presented in the same table. The results summarised in Table 3 have also been used to obtain values for $\log [K_{A1}{}^F - K_A]$ and $\log [K_{A2}{}^F - K_A]$ which are plotted against $\log\psi_{AB}$ in Figure 2.

Table 2 Comparison of paired estimates of $K_{A1}{}^F$ and $K_{A2}{}^F$ for muscarinic agonists acting against (−)-isoprenaline on guinea-pig isolated atria

Agonist A	*Number of experiments available*	*Number of experiments used*	*Number of times* $K_{A1}{}^F > K_{A2}{}^F$	*Probability (2-tailed)*	*Functional affinity constants* $K_{A1}{}^F$	$K_{A2}{}^F$
Pentyl-TMA	8	7	6	0.125	0.55 ± 0.17	0.41 ± 0.24
Methylfurmethide	9	5	3	0.50	11.3 ± 3.0	28.1 ± 24.7
Oxotremorine	3	1	1	1	133.6	62.4
Totals	20	13	10	0.09	—	—

Functional affinity constants are presented as means ± s.e., the units being (10^5 l/mol). Experiments were not used if either $K_{A1}{}^F$ or $K_{A2}{}^F$ was negative or was less than its own s.e.

Table 3 Comparison of various parameters estimated for muscarinic agonists acting on guinea-pig isolated atria

Agonist A	*Functional affinity constants estimated against (−)-isoprenaline*				*Affinity constants estimated using PrBCM*	*Comparison of agonist A with pentyl-TMA*	
	Tissues not pretreated with PrBCM		*Tissues pretreated with PrBCM*				
	K_{A1}^F	K_{A2}^F	K_{A1}^F	K_{A2}^F	K_A	ψ_{AB}	f_A/f_B
Pentyl-TMA	0.53 ± 0.15 (8)	0.41 ± 0.24 (7)	0.97 ± 0.34 (4)	0.16 ± 0.03 (4)	0.27 ± 0.09 (9)	1.0	1.0
Methylfurmethide	15.4 ± 3.3 (9)	28.1 ± 24.7 (5)	21.2 ± 16.2 (3)	1.26 ± 0.18 (4)	0.76 ± 0.19 (5)	27.8 ± 2.4 (3)	9.9
Oxotremorine	111 ± 47 (3)	62.4 (1)	—	—	8.4 ± 4.2 (3)	368 ± 33 (3)	11.8

Numbers in parentheses indicate the size of the sample on which each mean and s.e. is based. The units of all values of K_A^F and K_A are (10^5 1/mol). Individual values of K_{A1}^F or K_{A2}^F were excluded if they were negative or if they were less than their estimated s.e.

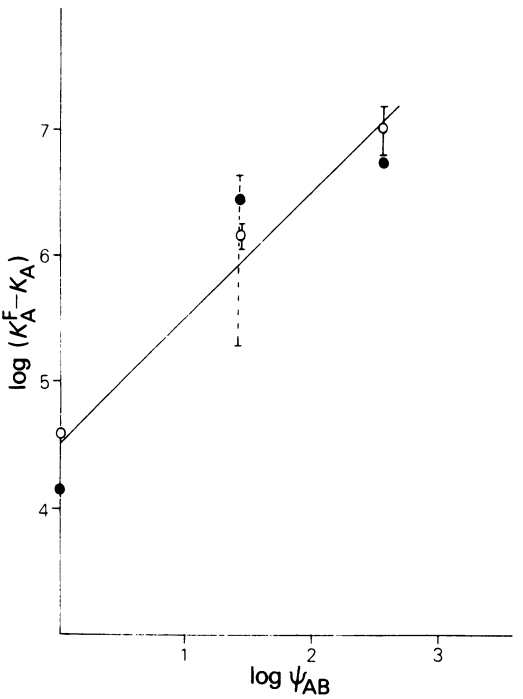

Figure 2 A plot of log $(K_A^F - K_A)$ against log ψ_{AB}. Values of log $(K_{A1}^F - K_A)$ and of log $(K_{A2}^F - K_A)$ are denoted respectively by the symbols (○) and (●). The dashed line is the best straight line with unit slope which can be fitted through all the points. The bars indicate s.e. mean. However, these are not inserted for pentyltrimethylammonium (for which log $\psi_{AB} = 0$) since in this case the closeness of the values of K_A^F and K_A leads to some very large standard errors for log $(K_A^F - K_A)$.

Discussion

The estimates that are presented in the Results section, of the affinity constants of atropine acting as an antagonist of pentyl-TMA, methylfurmethide and oxotremorine are clearly not significantly different, which suggests that all three agonists probably act on the same muscarinic receptors in the atria.

The results summarized in Table 1 show, on the basis of a sign test, that $(\alpha - 1)$ and γ differ significantly from zero for both pentyl-TMA and methylfurmethide. The same conclusion applies to the values of β for pentyl-TMA but not to those for methylfurmethide. The number of results available for oxotremorine is too small to permit any conclusions about the likely significance of α, β and γ for this agonist alone. However, if all three agonists act on the same receptors the overall conclusion must be that $(\alpha - 1)$, β and γ all differ from zero under appropriate experimental conditions. The functional antagonism of (−)-isoprenaline by these muscarinic agonists is therefore type I (see Table 1; Mackay, 1981). Although these results support the general form of equation 1 in preference to any simplified version of it, a clearer impression of how well the equation fits such data is best obtained by inspection of Figure 1a, 1b and 1c. It is worth noting that although the values of β in Figure 1b and 1c are not significantly different from zero when compared with the approximate estimates of their s.e.'s, the fit of the calculated points to the experimental curves is good in all three cases.

Before considering the results presented in Tables 2 and 3 two points need to be emphasised. The first is that the accurate estimation of α and γ or of α and β depends on the precise determination of the displacement and change in shape of the two curves at

either low or at high concentrations of A respectively. These are therefore the same requirements for the accurate estimation of K_{A1}^F and K_{A2}^F respectively. The optimum conditions for the determination of K_{A1}^F are therefore generally poorer conditions for the determination of K_{A2}^F, and *vice versa*. The second point is that there is a basic difference between K_A and K_A^F. The value of K_A would be expected to depend on the structures of the agonist and its receptor and as such should be a 'chemical constant' with a true value, though its estimation is subject to experimental error. By contrast, K_A^F depends not only on K_A but also on R_I, a_I and b_I. Since the latter might be expected to vary from one piece of tissue to another K_A^F is not a basic constant but has the characteristics of a population mean. These comments may have a bearing on the large s.e. associated with some values of K_{A1}^F and K_{A2}^F presented in Tables 2 and 3, although the smallness of the samples and the vagaries of sampling probably also contribute to the problem. However, regardless of the cause, mean values with large percentage s.e.'s will be considered less dependable in discussing the results.

Returning to the values of K_{A1}^F and K_{A2}^F summarized in Table 2, it will be seen that in general K_{A1}^F is greater than K_{A2}^F. However, the probability of obtaining such results by chance is not low enough to reach statistical significance (set at $P > 0.05$, 2 tailed sign test) for any one agonist but comes close to significance if the results for all three agonists are combined. If other results obtained on atria pretreated with PrBCM are also taken into account (see Table 3, $K_{A1}^F > K_{A2}^F$ 7 times out of 8) then the overall probability drops to $P < 0.01$ suggesting that there is a real difference between K_{A1}^F and K_{A2}^F although comparison of the mean values presented in Table 2 indicates that the difference may not be very great.

The intrinsic efficacies of methylfurmethide and oxotremorine relative to pentyl-TMA are both close to ten (Table 3) showing that pentyl-TMA has by far the lowest intrinsic efficacy of the three agonists. These results also imply that the difference in potency between methylfurmethide and oxotremorine is likely to be due mainly to the higher affinity of the latter for the muscarinic receptors in the atria.

It will be seen from Table 3 that for pentyl-TMA the values for K_{A1}^F and K_{A2}^F, with or without pretreatment of the tissue with PrBCM, do not generally differ greatly from the estimate of K_A. This result would be expected, on the basis of the model being tested here, for an agonist of low intrinsic efficacy acting by a type I mechanism. By contrast, the functional affinity constants for methylfurmethide and oxotremorine, which have much higher intrinsic efficacies than pentyl-TMA, tend to be 10 to 20 times greater than the corresponding affinity constants when estimated on atria not pretreated with PrBCM. For an agonist of high intrinsic efficacy it should be possible to reduce K_A^F towards K_A by reducing the density of receptors in the tissue (see equation 3). The value of K_{A2}^F obtained for methylfurmethide on atria pretreated with PrBCM tends to support this prediction.

According to equation 6 the discrepancy between K_A^F and K_A should be related to ψ_{AB} which for full agonists is their relative potency. The plot of $[\log K_A^F - K_A]$ against ψ_{AB}, where B is the reference compound pentyl-TMA, is in surprisingly good agreement with theory (see Figure 2) taking into account the small number of tissues in some of the samples and the difficulty of obtaining reproducible results.

The overal conclusion from these quantitative tests of the null equation for functional interaction is that the model on which it is based provides a reasonably good quantitative description of the interaction between (−)-isoprenaline and muscarinic agonists on guinea-pig isolated atria, this interaction being type I. The main discrepancy between the experimental results and the predictions of the model is that K_{A1}^F and K_{A2}^F are probably not equal. One possible explanation for this discrepancy is that the interaction may be mainly type I but with a type II component. Another possible explanation is that the equation

$$1/S_\Omega = a + b/S_\alpha$$

which was assumed to describe the relationship between the primary stimulus S_α and the final stimulus in the chain, S_Ω, is satisfactory over a limited range of stimulus but not over the entire range of possible stimuli. This would lead to different values of K_{A1}^F and K_{A2}^F since these are estimated from different regions of the state-concentration curves. This interpretation of the discrepancy would be in agreement with the fact that although K_{A1}^F and K_{A2}^F may not be equal they do tend to parallel each other (see Table 3 and Figure 2). The question may then be asked as to whether either of these values of K_A^F can be used to obtain information concerning sites of action of functional interactants, as was suggested in the preceding paper. The answer to this question is probably in the affirmative since such studies merely require information about the relative magnitudes of K_A^F estimated for two functional antagonists or synergists interacting with the same chosen agonist. Such comparisons should be possible by studying both interactants on the same piece of tissue and comparing either values of K_{A1}^F or values of K_{A2}^F depending on which of these can be estimated with the greater precision.

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