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Application of the operational model of agonism to establish conditions when functional antagonism may be used to estimate agonist dissociation constants

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1 The operational model of agonism (Black & Leff, 1983) has been used to analyse comparatively functional antagonism and irreversible antagonism as methods for estimating agonist dissociation constants (K_As).

2 A general condition is established in terms of the model parameters which defines the type of experimental interventions at the receptor and the post-receptor level that allow valid K_A estimation.

3 It is shown that functional antagonism and other post-receptor interventions may produce changes in agonist-concentration effect curves which are qualitatively indistinguishable but quantitatively distinct, from those produced by irreversible antagonism.

4 Experimental data obtained with the guinea-pig tracheal strip preparation are in keeping with the theoretical predictions and show how studies using functional antagonism may overestimate agonist affinity.

5 In general, functional antagonism, unlike irreversible antagonism, is in principle an unreliable method for quantifying agonism.

Introduction

The agonist activity of hormones and drugs is a function of two factors, affinity and efficacy (Stephenson, 1956; Furchgott, 1966; Colquhoun, 1973; Kenakin, 1983). Quantification of both properties provides the basis for classifying agonist action and complements the use of competitive antagonists in the classification of receptors (Jenkinson, 1978; Black & Leff, 1983). Both affinity and efficacy are properties of AR, the complex formed by the agonist, A, and its receptor, R. Affinity is defined by the reciprocal of the dissociation constant, K_A, for AR; efficacy is defined by the ability of AR to elicit a response. Thus affinity is a chemical quantity relating only to A and R, while efficacy is an operational quantity, depending in addition on subsequent events in the tissue (Black & Leff, 1983; Kenakin, 1983).

It is, in principle, possible to quantify both the affinity and efficacy of agonists using so-called null methods (Stephenson, 1956; Furchgott, 1966; Mackay, 1966; Barlow *et al.*, 1967). These methods require no knowledge of intracellular events. For example, the application of a specific irreversible antagonist (Furchgott, 1966) can be assumed simply to reduce the functional receptor concentration, so that equal agonist effects in the presence and absence of the antagonist reflect equal concentrations of AR. It has also been suggested that functional antagonism (Buckner & Saini, 1975; Broadley & Nicholson, 1979) and other post-receptor interventions (Gião T. Rico, 1971a,b) may be used to quantify agonism. However, these procedures deliberately change the transducer machinery and there seems to be no *a priori* reason why they should allow reliable quantification of agonist affinity and efficacy. The theoretical basis for the functional antagonism method has been explored (Mackay, 1981; Amidon & Buckner, 1982) showing that a 'null' equation may be developed which is formally equivalent to that which allows estimation of the K_A in the case of irreversible antagonism. However, as Mackay (1981) has shown, this null equation applies in only one of several different types of functional antagonism and generally, accurate estimation of K_A is not possible.

In this paper, rather than adopting the conventional 'null' approach to the quantification of agonism, we seek to complement such analyses with an approach based on a recently developed 'operational' model of agonism (Black & Leff, 1983). This model provides an

explicit description of agonist concentration-effect (E/[A]) curves, allowing the problem to be approached in terms of predicted E/[A] curve profiles and the changes in them resulting from different types of experimental interventions. Initially, the basis of the irreversible antagonism method (Furchgott, 1966) is analysed in these terms. This analysis allows a general condition to be established for K_A estimation by intervention at the receptor or post-receptor level. Next, circumstances are identified where functional antagonism obeys this general condition. Experimental data comparing irreversible and functional antagonism are then presented and analysed in these terms.

Theory: the operational model of agonism

The model, developed elsewhere (Black & Leff, 1983), defines the relation between agonist concentration, [A], and pharmacological effect, E, as follows:

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (1)$$

in which K_A is the dissociation constant for AR, the agonist-receptor complex, E_m is the maximum possible effect, and τ is the operational efficacy of the agonist. τ is defined by the ratio $[R_o]/K_E$ where $[R_o]$ is the total function receptor concentration and K_E is the value of [AR] for half E_m. Thus, K_E determines the efficiency of the transducer function relating [AR] to E. n is the slope parameter for the transducer function (see Black & Leff, 1983).

General conditions for K_A estimation

Estimation of K_A and the other parameters in the model can be achieved by fitting equation (1) directly to experimental E/[A] data as demonstrated in a previous analysis (Black *et al.*, 1985). Experimental E/[A] curves are described by three parameters; an asymptote (α), a location ($[A_{50}]$) and a mid-point gradient (G) which are defined by the model as follows:

$$\alpha = \frac{E_m \tau^n}{1 + \tau^n} \quad (2)$$

$$[A_{50}] = \frac{K_A}{((2 + \tau^n)^{1/n} - 1)} \quad (3)$$

$$G = \frac{0.576n(2 + \tau^n)((2 + \tau^n)^{1/n} - 1)}{(2 + \tau^n)^{1/n}(1 + \tau^n)} \quad (4)$$

If α can be estimated reliably as a fraction of E_m then τ^n can be calculated. G provides an estimate of n which allows K_A to be estimated from $[A_{50}]$. When τ is very large ($\tau \gg 1$), a condition which defines full agonism, α is not significantly less than E_m and the estimation of K_A is not possible. Under these circumstances, τ must be reduced experimentally to a value in the region of unity which allows α to be estimated and therefore K_A to be calculated. In model terms this is how the irreversible antagonism method (Furchgott, 1966) works, the irreversible ligand decreasing $[R_o]$ and, therefore, reducing τ.

Variation in τ with the other model parameters fixed leads to the familiar rightward shift and depression of E/[A] curves. These changes are characterised by the relation between α and $[A_{50}]$ which is derived by eliminating τ between equations (2) and (3) giving:

$$\alpha = E_m \left(1 - \frac{1}{(K_A/[A_{50}] + 1)^n - 1} \right) \quad (5)$$

As τ is reduced towards zero, α approaches zero and $[A_{50}]$ approaches a value which defines K_A. Figure 1 shows that, for rectangular hyperbolic E/[A] curves (n = 1), $[A_{50}]$ approaches K_A identically; for 'shallow' E/[A] curves (n < 1), the asymptotic $[A_{50}]$ undershoots K_A and for 'steep' E/[A] curves (n > 1) the asymptote overshoots K_A.

In model terms, therefore, the general basis for K_A estimation is the characterisation of E/[A] curve-shape dependence on τ.

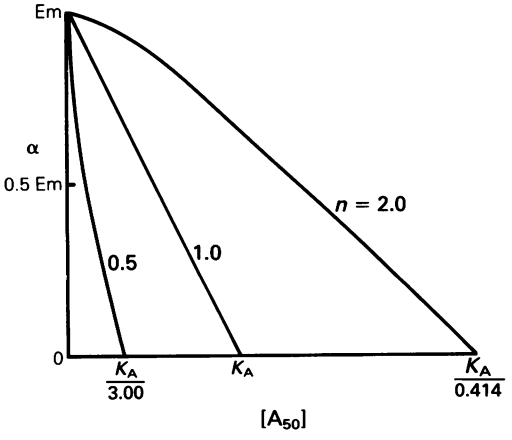


Figure 1 Correlation between α and $[A_{50}]$ with varying τ for hyperbolic and non-hyperbolic E/[A] curves: the α/$[A_{50}]$ plots were constructed according to model equation (5) for n at 0.5, 1.0 and 2.0. When n = 1 the plot is a straight line which intersects the ordinate and abscissa at E_m and K_A respectively. When n < 1 the abscissal intercept is less than K_A; when n > 1 the intercept is greater than K_A.

Post-receptor interventions and K_A estimation

The dependence of τ on K_E as well as $[R_o]$ implies that the estimation of K_A might also be possible using experimental conditions which increase K_E e.g. functional antagonism. However, there is no reason *a priori* to anticipate that such an intervention will affect only K_E. When considering irreversible antagonism the nature of the transducer relation is not an issue, so long as it is operationally fixed. In fact this relation is likely to consist of a cascade of saturable functions (Furchgott, 1966; Mackay, 1981; Amidon & Buckner, 1982). When considering post-receptor interventions, the level in this cascade at which the intervention takes place becomes an issue. This can be illustrated by considering the simple case of a rectangular hyperbolic transducer (E/[AR]), relation made up of two sequential rectangular hyperbolic relations. The first relation can be taken to represent the saturable production of an intracellular mediator, M (e.g. cyclic AMP or Ca^{2+}):

$$[M] = \frac{a[AR]}{b + [AR]} \tag{6}$$

in which **a** is the maximum concentration of M that can be produced by occupied receptors and **b** is the value of [AR] for half **a**. The second relation then links M to pharmacological effect:

$$E = \frac{p[M]}{q + [M]} \tag{7}$$

in which **p** is the maximum value of E that M can produce, and **q** is the value of M for half **p**.

The overall transducer function then consists of (6) substituted into (7) giving:

$$E = \frac{p\ a\ [AR]}{bq + (a + q)[AR]} \tag{8}$$

The general form of the transducer function in the model is

$$E = \frac{E_m[AR]}{K_E + [AR]} \tag{9}$$

Comparison of equation (8) with equation (9) shows that:

$$E_m = pa/(a + q) \tag{10}$$
$$K_E = bq/(a + q) \tag{11}$$

Functional antagonism may, in principle, affect any of the parameters **a**, **b**, **p** and **q**. Variation in **p** alone causes variation in E_m but not K_E; variation in **a** or **q** leads to concomitant variation of E_m and K_E; variation in **b** alone causes variation in K_E but not E_m. Therefore, **a** 'pure' K_E change is anticipated only for variation in **b**, that is, for a change in the location parameter of the first saturable relation in the transducer function. This result is true regardless of the number of hyperbolic relations linked in sequence.

Thus, a post-receptor intervention of the kind required to estimate K_A cannot be guaranteed or anticipated. More importantly, it may be impossible to detect that the 'wrong' kind of intervention has been made and that the estimate of K_A is consequently erroneous. Making the following definitions:

$$\tau_M = [R_o]/b \tag{12}$$
$$\tau_E = a/q \tag{13}$$

allows the E/[A] relation to be written:

$$E = \frac{p\tau_M\tau_E[A]}{K_A + (1 + \tau_M(1 + \tau_E))[A]} \tag{14}$$

τ_M and τ_E are the individual transducer ratios that determine respectively, the efficiency with which occupancy is coupled to M production and M is coupled to pharmacological effect, E. Overall, $\tau = \tau_M(1 + \tau_E)$.

The asymptote and location parameters for equation (14) are now:

$$\alpha = \frac{p\tau_M\tau_E}{1 + \tau_M(1 + \tau_E)} \tag{15}$$

$$[A_{50}] = \frac{K_A}{1 + \tau_M(1 + \tau_E)} \tag{16}$$

The product $\tau_M\tau_E$ can be eliminated between equations (15) and (16) giving:

$$\alpha = p\left(1 - \frac{[A_{50}](1 + \tau_M)}{K_A}\right) \tag{17}$$

Equation (17) defines the relationship between α and $[A_{50}]$ with variation in τ_E at fixed τ_M. Eliminating τ_M between (15) and (16) allows the relation between α and $[A_{50}]$ with variation in τ_M to be defined at fixed τ_E:

$$\alpha = p\frac{\tau_E}{(1 + \tau_E)}(1 - [A_{50}]/K_A) \tag{18}$$

Figure 2 illustrates equations (17) and (18) graphically. Evidently, as τ_M is reduced at fixed τ_E, $[A_{50}]$ approaches K_A. This is expected as τ is proportional to

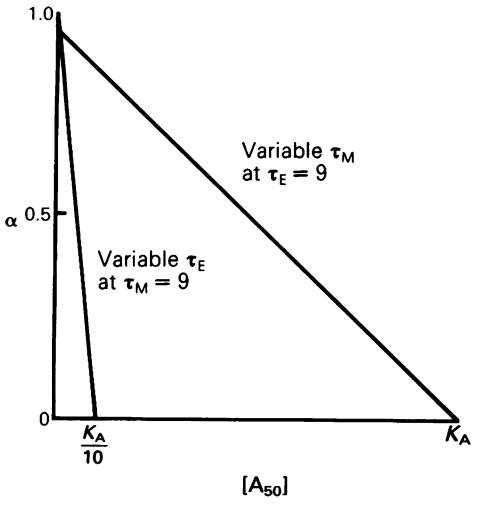


Figure 2 Relation between asymptote and $[A_{50}]$ for different types of post-receptor intervention: the $\alpha/[A_{50}]$ plots were constructed according to model equations (17) and (18). These, respectively, predict the correlation between α and $[A_{50}]$ for hyperbolic E/[A] curves: (i) when the functional intervention occurs immediately post-receptor (that is, τ_M is altered); (ii) when the intervention occurs at a subsequent step in the transducer machinery (that is, τ_E is altered). In case (i) the correlation can be used to estimate K_A whereas in case (ii) the correlation underestimates K_A.

τ_M and it has already been shown (see Figure 1 and accompanying analysis) that (for hyperbolic E/[A] curves) when τ is reduced $[A_{50}]$ approaches K_A in the limit. In contrast, as τ_E is reduced at fixed τ_M, $[A_{50}]$ approaches the value $K_A/(1 + \tau_M)$. Significantly, the relationship between α and $[A_{50}]$ is of the same linear form in both cases, differing only in the asymptotic $[A_{50}]$ value as α tends to zero. Without prior knowledge of K_A it is impossible to distinguish between the two situations. However, in only one of the cases, that is τ_M variation, does the relationship between α and $[A_{50}]$ furnish a true K_A.

Therefore, post-receptor interventions can be predicted that cause E/[A] curve changes which are indistinguishable from simple $[R_o]$ or K_E changes, but which do not allow K_A estimation. In general, underestimation of K_A is predicted when the intervention is beyond the first saturable step in the transducer function.

Methods

Preparation of guinea-pig tracheal strips

Male albino guinea-pigs (Dunkin Hartley; 400–450 g) were treated with 6-hydroxydopamine (50 mg kg^{-1}, i.v.) 48 h and 24 h before they were killed, in order to prevent possible interference from the release of endogenous catecholamines (O'Donnell & Saar, 1974). Each animal was killed by a blow to the head, the entire trachea excised and three zig-zag tracheal strips of equal size prepared using the procedure described by Emmerson & Mackay (1979). Strips were rapidly transferred to 20 ml organ baths containing modified Krebs solution of the following composition (mM); NaCl 118.4, $NaHCO_3$ 25.0, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11.1, $CaCl_2$ 2.5, ascorbic acid 0.28 and Na_2EDTA 0.04. This was maintained at 37°C and continually gassed with 95% O_2: 5% CO_2. Indomethacin (2.8×10^{-6} M) was included to prevent the development of smooth muscle tone due to synthesis of cyclo-oxygenase products. Each tracheal strip was attached by cotton thread to a rotary transducer (Schaevitz R30-D) and subject to a passive load of 0.5 g. Tissues were allowed to attain a steady resting length prior to starting the experiment. Responses to drug additions were measured as isotonic changes in tissue length and recorded on Rikadenki flat-bed potentiometric recorders.

Experimental protocols

Irreversible inactivation of receptors Tracheal strips were challenged with a near-maximally effective concentration of histamine (10^{-5} M) to establish viability and provide a reference contracture that was used to normalize subsequent responses to 5-methylfurmethide (5-MeF). After washout of the histamine, tissues were incubated with phenoxybenzamine (Pbz) (10^{-6} M or 10^{-5} M) for 40 min after which excess inhibitor was removed by several changes of the organ bath Krebs solution. Full E/[A] curves were then established by cumulative additions of 5-MeF, the agonist concentration increasing in 0.5 lg unit increments.

Functional antagonism After the challenge with histamine (10^{-5} M), tissues were exposed to (±)-isoprenaline (Iso) (10^{-7} M or 10^{-4} M) for 20 min before and during construction of 5-MeF E/[A] curves.

For both of these procedures, only a single E/[A] curve was generated in each tissue. Each animal provided three tissues, one of which served as a control, the other two for treatments.

Data analysis

All the following fitting procedures were unweighted, iterative least squares minimization computer programmes. They were locally written with the exception of BMDP Module AR (BMDP Statistical Software, 1981) which was the programme used to fit data to the model of agonism.

Logistic-fitting

Individual sets of 5-MeF E/[A] curve data were fitted to a logistic function of the form:

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m}$$

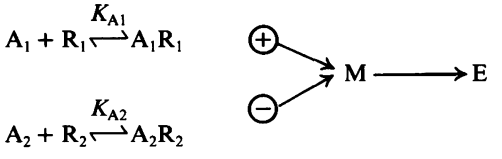
in which α, $[A_{50}]$ and m are the asymptote, location and slope parameters respectively. Location parameters were actually estimated as logarithms by making the substitution $[A_{50}] = 10^{lg}[A_{50}]$. The asymptote and location parameters quoted and analysed in the text are these computed estimates.

Model fitting

Each set of three 5-MeF E/[A] curves was fitted to model equation (1) providing direct estimates of E_m, n and K_A, and three estimates of τ, one for each curve in a set. Goodness-of-fit was assessed by examining fitted and experimental data points for systematic deviations.

Simulation of Mackay's model of functional antagonism

The interaction between Iso and 5-MeF was interpreted using Mackay's (1981) Type I model of functional antagonism, which can be represented schematically as follows:



in which A_1 is 5-MeF and A_2 is Iso in the present example. A_1 binds to receptors R_1 forming a complex, A_1R_1, which stimulates the production of a response mediator, M. A_2 binds to receptors R_2 forming a complex, A_2R_2 which depletes M. Effect, E, is saturably related to the net concentration of M.

To simulate experimental data, the $[M]/[A_1R_1]$ and $[M]/[A_2R_2]$ functions were assumed to be hyperbolic. Therefore, the net concentration of M can be represented by:

$$[M] = \frac{M_1\tau_{M1}[A_1]}{K_{A1} + (1 + \tau_{M1})[A_1]} - \frac{M_2\tau_{M2}[A_2]}{K_{A2} + (1 + \tau_{M2})[A_2]}$$

M_1 and M_2 are the maximal production and depletion of M by A_1 and A_2 respectively.

Also, in order to account for the slope of the control 5-MeF curve, the E/[M] function was assumed to be of logistic form, that is:

$$E = \frac{[M]^n}{K^n + [M]^n}$$

Drugs

The following drugs (source in parentheses) were used: atropine sulphate (Sigma); 6-hydroxydopamine hydrobromide (Sigma); indomethacin (Sigma); (±)-isoprenaline sulphate (Sigma); 5-methylfurmethide iodide (Wellcome Research Laboratories); phenoxybenzamine hydrochloride (Dibenyline: Smith, Kline and French). Solutions of all drugs were prepared in fresh physiological medium or distilled water. Immediately before administration to animals, 6-hydroxydopamine was dissolved in sterile saline solution (0.85% w/v) containing ascorbic acid (2% w/v).

Results

Figure 3 illustrates the typical effects of 0, 10^{-6}M and 10^{-5}M Pbz on 5-MeF E/[A] curves. The lines through the data in Figure 3 are the results of model-fitting. They typify the general goodness-of-fit of the model, although some systematic deviations were evident at threshold agonist concentrations. This may be attributable to the presence of indomethacin which has been reported to compromise the contractile

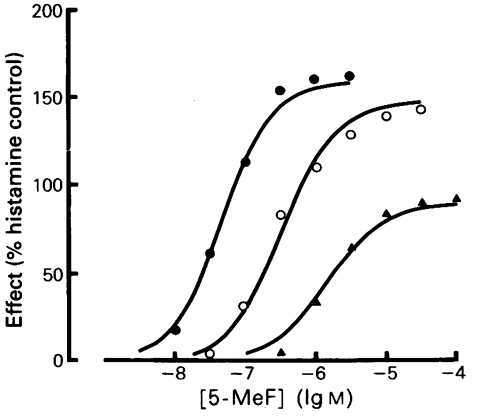


Figure 3 Effect of phenoxybenzamine (Pbz) on 5-methylfurmethide (5-MeF) E/[A] curves: the diagram shows one of five sets of 5-MeF E/[A] curves obtained in the three tissues from a single animal at 0 (●), 10^{-6}M (○) and 10^{-5}M (▲) Pbz. The lines through the data were produced by model-fitting, using equation (1) (see text). The model parameter estimates for these data are those given in Table 1 for animal 4.

Table 1 Operational model fitting of 5-methylfurmethide E/[A] curves in the presence and absence of phenoxybenzamine (Pbz)

Animal	E_m	pK_A	n	τ_1	τ_2	τ_3
1	170.9	5.56	1.01	223.9	7.1	0.85
2	188.1	6.01	1.05	15.5	1.7	0.28
3	160.5	5.67	1.29	46.8	6.8	1.23
4	159.2	5.37	1.40	102.3	12.4	0.83
5	143.1	5.50	1.30	89.1	2.5	0.47

Each animal provided three tissues in which a control E/[A] curve, a 10^{-6}M Pbz treatment curve and a 10^{-5}M Pbz treatment curve was obtained. Each set of three E/[A] curves were fitted to equation (1). Values of E_m, K_A and n were estimated for each set together with three τ values, τ_1, τ_2 and τ_3 corresponding, respectively, to the control, 10^{-6}M and 10^{-5}M Pbz treatment curves. K_A values were estimated as the negative logarithms (pK_A).

responses of guinea-pig trachea to low concentrations of various stimulants including acetylcholine (Orehek *et al.*, 1975). Table 1 summarizes the estimates of K_A, E_m, τ and n obtained by model-fitting the data from each of five replicate experiments. The average estimate of pK_A was 5.62 ± 0.11 (mean ± s.e., $n = 5$). Variation in the control τ values, that is τ_1, may reflect between-animals differences in $[R_o]$.

Figure 4 shows the protective action of concomitant atropine incubation against the effects of Pbz. For control curves, atropine (10^{-7}M) treatment curves and atropine (10^{-7}M)/Pbz (10^{-5}M) treatment curves, asymptotes were not significantly different. Also the locations of the atropine and atropine/Pbz treatment curves were not different ($p[A_{50}]$ after atropine = 7.15 ± 0.05; mean ± s.e., $n = 7$; $p[A_{50}]$ after atropine/Pbz = 7.05 ± 0.05; mean ± s.e., $n = 7$), although both groups were slightly rightward shifted from the control position ($p[A_{50}] = 7.50 \pm 0.09$; mean ± s.e., $n = 7$) indicating that some atropine antagonism remained after washing for 120 min.

The functional antagonism of 5-MeF by Iso is shown in Figure 5. In the presence of Iso, 5-MeF E/[A] curves were steepened then depressed; at concentra-

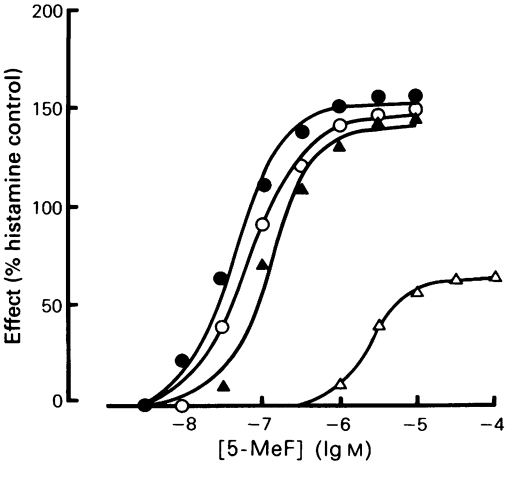


Figure 4 Effect of concomitant atropine incubation on phenoxybenzamine (Pbz) induced irreversible antagonism of 5-methylfurmethide (5-MeF) responses: representative 5-MeF E/[A] curves are shown: control (●); atropine (10^{-7}M) then washout (○); Pbz (10^{-5}M) then washout (△); atropine (10^{-7}M) and Pbz (10^{-5}M) then washout (▲). The lines through the data are logistic fits.

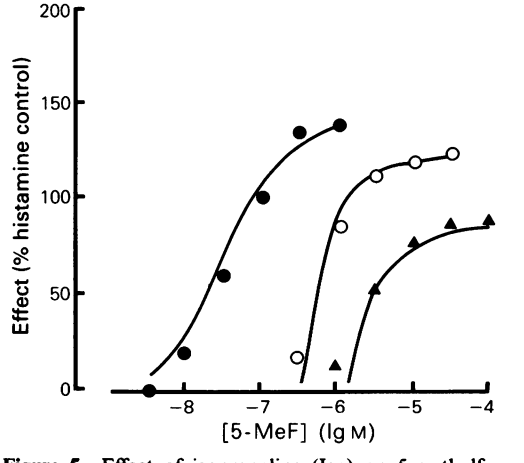


Figure 5 Effect of isoprenaline (Iso) on 5-methylfurmethide (5-MeF) E/[A] curves: the diagram shows a typical set of 5-MeF E/[A] curves obtained in the three tissues of a single animal at 0 (●), 10^{-7}M (○) and 10^{-4}M (▲) Iso. The lines drawn through the data were obtained by simulating Mackay's Type I model of functional antagonism (Mackay, 1981) (see Methods).

The simulated lines were obtained with the following parameter values: $M_1 = 100$; $M_2 = 96$; $K_{A1} = 3 \times 10^{-6}$; $K_{A2} = 2 \times 10^{-7}$; $\tau_{M1} = 10$; $\tau_{M2} = 4$; $K = 12$; n = 1.2. Note that the K_{A1} and K_{A2} values used accord with the suspected true dissociation constants for 5-MeF and Iso respectively (see text).

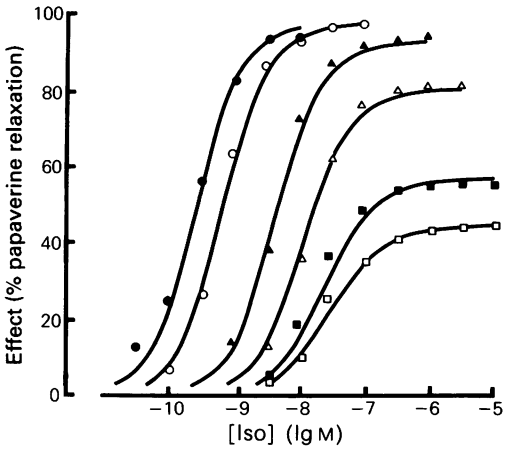


Figure 6 Effect of carbamylcholine on isoprenaline (Iso) E/[A] curves (redrawn from Buckner & Saini (1975): the data points were measured from the published diagram (Buckner & Saini, 1975) and fitted using equation (1), producing the lines shown. Only τ was allowed to vary between the six E/[A] curves. The estimated parameter values were: $E_m = 97$; $pK_A = 7.46$; $n = 1.32$; $\tau_1 = 140$; $\tau_2 = 54$; $\tau_3 = 9.3$; $\tau_4 = 3.2$; $\tau_5 = 1.3$; $\tau_6 = 0.9$.

tions above 10^{-6}M, Iso failed to cause further right-shift and depression. Since the results clearly do not conform to the expectations for a reduction in τ by a simple increase in K_E, no attempt was made fit model equation (1) to the data. However, these data were compatible with Mackay's (1981) model of functional antagonism involving the subtraction of opposing effects produced by the agonist and the functional antagonist. The lines drawn through the data were obtained by simulating the Type 1 variant of this model (see Methods).

Unlike the interaction between Iso and 5-MeF there are examples in the literature where functional antagonism produces effects apparently indistinguishable from irreversible antagonism. One such example was produced by Buckner & Saini (1975) for the functional antagonism of Iso responses by carbamylcholine. The data, reproduced in Figure 6, show the rightward displacement and ultimate depression of Iso E/[A] curves by increasing concentrations of muscarinic agonist. The lines through these data were obtained by fitting them to the model (equation (1)), allowing τ to vary between curves; the data are apparently consistent with simple τ variation. From this analysis, the K_A was estimated to be 3.5×10^{-8}M; the other parameter estimates are shown in the Figure legend. The validity, or otherwise, of this K_A estimate is considered below.

Discussion

The operational model (Black & Leff, 1983) defines agonist action by four parameters; K_A, τ, E_m and n. The present analysis shows that agonist dissociation constants can be estimated if τ can be varied experimentally with the other parameters fixed. In general, any agent or experimental intervention which produces a simple τ reduction may be used. The definition of τ as $[R_o]/K_E$ conveniently classifies the types of experimental interactions which allow K_A estimation. Irreversible antagonists work simply by reducing $[R_o]$ at fixed K_E. A functional antagonist which simply produces an increase in K_E may also be used. However, a functional antagonist may also affect other parameters of agonism, leading to erroneous K_A estimation. Practically, in order to check whether a functional antagonist produces a simple K_E change an irreversible antagonist must be available for comparison.

In the present experimental study it was evident that the effects of Iso on 5-MeF E/[A] curves were not congruent with the effects produced with Pbz, either qualitatively or quantitatively (Figures 3 and 5). In fact the kind of functional antagonism obtained resembled that reported by Van den Brink (1973) for the interaction between Iso and methacholine in calf tracheal muscle, which is consistent with Mackay's (1981) modified form of Van den Brink's model (1973) as shown in Figure 5. This type of functional interaction generally does not permit K_A estimation, (Mackay, 1981).

In other experimental systems, for example β-adrenoceptor systems, functional antagonism has been used in an attempt to quantify agonist activity at receptors for which an irreversible antagonist is not yet available. In some instances, functional antagonism produced effects on β-adrenoceptor E/[A] curves which were apparently indistinguishable from a simple decrease in τ (e.g. Buckner & Saini, 1975; Broadley & Nicholson, 1979; Broadley & McNeill, 1983). This is exemplified in Figure 6 by the data of Buckner & Saini (1975). Originally these data were analysed by Furchgott's method (1966) assuming equivalence between irreversible and functional antagonism, giving an estimate of K_A for Iso of 3×10^{-8}M. The inability of Iso to distinguish β_1- and β_2-adrenoceptors, both of which occur in the guinea-pig trachea (Furchgott & Wakade, 1975), means that the K_A estimate applies to both sub-types. Direct model-fitting using equation (1) estimated K_A to be 3.5×10^{-8}M. Evidently, the model fits the data acceptably well (consistent with Iso's non-selectivity) but, as discussed previously, this is not a sufficient criterion by which to judge the accuracy of the K_A estimate. For example, a considerable discrepancy exists between this estimate of K_A and that obtained by radioligand binding studies. Thus,

binding studies using cat cardiac or soleus muscle (Kaumann, 1978; Hedberg & Mattson, 1981), rat lung (Minneman *et al.*, 1979) and human cardiac muscle (Stiles *et al.*, 1983), yield an average estimate of K_A of 2×10^{-7}M. If this value is take to represent the 'true' K_A, it must be concluded that the functional antagonism method overestimates agonist affinity in this instance, a result which is consistent with concomitant E_m and τ reduction. Of course, there is no certainty that binding studies provide estimates of K_A which accurately represent agonist affinity for receptors in intact tissues. However, there is other evidence that 3×10^{-8}M is an overestimate of Iso affinity. In the guinea-pig isolated left atrium and extensor digitorum longus preparations Iso produces full agonism (Kenakin & Beek, 1980; Johansson & Persson, 1983) characterized by a rectangular hyperbolic E/[A] curve with an $[A_{50}]$ of between 10^{-8}M and 2×10^{-8}M. If the K_A were 3×10^{-8}M then Iso would be expected to demonstrate partial agonism; the E/[A] curve asymptote would have been only 15–30% of E_m (calculated using equations (2) and (3)).

Another kind of post-receptor experimental intervention which has been claimed to produce effects congruent with $[R_o]$ changes is extracellular Ca^{2+} variation. Gião T. Rico showed that, for both muscarinic (1971a) and histamine (H_1) (1971b) receptor agonists acting on the guinea-pig isolated ileum, depletion of extracellular Ca^{2+} mimicked irreversible antagonism. Considering Ca^{2+} as the intracellular mediator, M, in the system analysed in the text, a depletion in extracellular Ca^{2+} might be anticipated to lead to a concomitant τ and E_m change by a reduction in the asymptote (**a**) of the M/[AR] relation in the model. However, if the M/[AR] relation were effectively linear in the range of AR generated, (that is **b** >> [AR]) then any change in **a** would mimic a change in **b** (see equation (6)), a situation which would produce a pure change in K_E and, therefore, τ. Therefore, the congruence between Ca^{2+} depletion and $[R_o]$ reduction is an indicator of apparently linear coupling between AR and intracellular Ca^{2+}, assuming that the latter is proportional to the extracellular Ca^{2+} concentration. However, this is a feature of the particular agonist-receptor-tissue combination used; there is no guarantee that Ca^{2+} depletion would have the same consequences in other systems in which, for example, the $[Ca^{2+}]/[AR]$ relation is saturable.

The conclusion from the present analysis is that functional antagonism and other experimental post-receptor interventions yield valid estimates of K_A only when the intervention produces a change in the transducer function which is congruent with a decrease in $[R_o]$, that is, an increase solely in K_E. A concomitant reduction in E_m, which may not be detectable, is predicted to lead to an overestimation of affinity. It follows that the validity of functional antagonism as a method for K_A estimation can only be tested when an irreversible antagonist is available in the same system. This places heavy reliance on irreversible antagonism. However, a criterion exists for the classification of irreversible ligands which is unavailable in the case of functional antagonists, namely, protection by a reversible competitive antagonist (Ariëns *et al.*, 1964; Furchgott, 1966). In the present study the interaction between atropine and Pbz fulfilled this criterion confirming that the haloalkylamine acted purely to reduce $[R_o]$ in the system. It follows that the estimate of pK_A for 5-MeF is reliable.

References

AMIDON, G.L. & BUCKNER, C.K. (1982). A theoretical model for the use of functional antagonism to estimate dissociation constants for agonists. *J. Pharmac. Methods.*, **7**, 173–178.

ARIËNS, E.J., SIMONIS, A.M. & VAN ROSSUM, J.M. (1964). Drug-Receptor interaction: interaction of one or more drugs with one receptor system. In *Molecular Pharmacology*. ed. Ariëns, E.J. pp. 119–286. New York, San Francisco, London: (Academic Press).

BARLOW, R.B., SCOTT, N.C. & STEPHENSON, R.P. (1967). The affinity and efficacy of onium salts on the frog rectus abdominis. *Br. J. Pharmac. Chemother.*, **31**, 188–196.

BLACK, J.W. & LEFF, P. (1983). Operational models of pharmacological agonism. *Proc. R. Soc. B.*, **220**, 141–162.

BLACK, J.W., LEFF, P. & SHANKLEY, N. (1985). An operational model of pharmacological agonism: the effect of E/[A] curve shape on agonist dissociation constant estimation. *Br. J. Pharmac.*, **84**, 561–571.

BMPD Statistical Software (1981). ed. Dixon, W.J. Berkeley, Los Angeles and London: University of California Press.

BROADLEY, K.J. & NICHOLSON, C.D. (1979). Functional antagonism as a means of determining dissociation constants and relative efficacies of sympathomimetic amines in guinea-pig isolated atria. *Br. J. Pharmac.*, **66**, 397–404.

BROADLEY, K.J. & McNEILL, J.H. (1983). Dissociation constants (K_A) and relative efficacies of sympathomimetic amines in isolated atria during hypothermia-induced supersensitivity. *Can. J. Physiol. Pharmac.*, **61**, 572–580.

BUCKNER, C.K. & SAINI, R.K. (1975). On the use of functional antagonism to estimate dissociation constants for beta adrenergic receptor agonists in isolated guinea-pig trachea. *J. Pharmac. exp. Ther.*, **194**, 565–574.

COLQUHOUN, D. (1973). The relation between classical and co-operative models for drug action. In *Drug Receptors*. ed. Rang, H.P., pp. 149–182. London: MacMillan.

EMMERSON, J. & MACKAY, D. (1979). The zig-zag tracheal strip. *J. Pharm. Pharmac.*, **31**, 798.

FURCHGOTT, R.F. (1966). The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. In *Advances in Drug Research*. ed. Harper, N.J. & Simmonds, A.B. pp. 21–55. London: Academic Press.

FURCHGOTT, R.F. & WAKADE, T.D. (1975). Evidence for both β_1 and β_2 receptors in guinea-pig tracheal smooth muscle and variation of the β_1:β_2 ratio in different animals. In *Abstracts 6th Int. Cong. Pharmac.*, Helsinki. p. 622.

GIÃO T. RICO, J.M. (1971a). The influence of calcium on the activity of full and partial muscarinic agonists. *Eur. J. Pharmac.*, **13**, 218–229.

GIÃO T. RICO, J.M. (1971b). The determination of the histamine equilibrium constant for its receptor in the smooth muscle by changing the extracellular calcium concentration. *Eur. J. Pharmac.*, **16**, 209–213.

HEDBERG, A. & MATTSON, H. (1981). Beta adrenoceptor interaction full and partial agonists in cat heart and soleus muscle. *J. Pharmac. Exp. Ther.*, **219**, 789–808.

JENKINSON, D.H. (1978). Partial agonists in receptor classification. *Proceedings of the International Symposium on Medicinal Chemistry*. ed. Simpkins, M.A. pp. 373–383. Oxford: Cotswold Press.

JOHANSSON, L-H & PERSSON, H. (1983). β_2-Adrenoceptors in guinea-pig atria. *J. Pharm. Pharmac.*, **36**, 804–807.

KAUMANN, A.J. (1978). On spare β-adrenoceptors for inotropic effects of catecholamines in kitten ventricle. *Naunyn-Schmiedebergs Arch. Pharmac.*, **305**, 97–102.

KENAKIN, T.P. (1983). Receptor classification by selective agonists: coping with circularity and circumstantial evidence. *Trends Pharmac. Sci.*, **4**, 291–295.

KENAKIN, T.P. & BEEK, D. (1980). Is prenalterol (H133/80) really a selective $beta_1$-adrenoceptor agonist? Tissue selectivity resulting from differences in stimulus-response relationships. *J. Pharmac. Exp. Ther.*, **213**, 406–413.

MACKAY, D. (1966). A new method for the analysis of drug-receptor interactions. *Adv. Drug Res.*, **3**, 1–19.

MACKAY, D. (1981). An analysis of functional antagonism and synergism. *Br. J. Pharmac.*, **73**, 127–134.

MINNEMAN, K.P., HEGSTRAND, L.R. & MOLINOFF, P.B. (1979). Simultaneous determination of $beta_1$- and $beta_2$-adrenergic receptors in tissues containing both receptor sub-types. *Mol. Pharmac.*, **16**, 34–46.

O'DONNELL, S.R. & SAAR, N. (1974). The effects of 6-hydroxydopamine and guanethidine on peripheral adrenergic nerves in the guinea-pig. *Eur. J. Pharmac.*, **28**, 251–256.

OREHEK, J., DOUGLAS, J.S. & BOUHOYS, A. (1975). Contractile responses of the guinea-pig trachea *in vitro*: modification by prostaglandin synthesis-inhibiting drugs. *J. Pharmac. exp. Ther.*, **194**, 554–564.

STEPHENSON, R.P. (1956). A modification of receptor theory. *Br. J. Pharmac. Chemother.*, **11**, 379–393.

STILES, G.L., TAYLOR, S. & LEFKOWITZ, R.J. (1983). Human cardiac beta-adrenergic receptors: sub-type heterogeneity delineated by direct radioligand binding. *Life Sci.*, **33**, 467–473.

VAN DEN BRINK, F.G. (1973). The model of functional interaction. I. Development and first check of a new model of functional synergism and antagonism. *Eur. J. Pharmac.*, **22**, 270–278.

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