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Analysis of competitive antagonism when this property occurs as part of a pharmacological resultant

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1 In this paper, pharmacological resultant is defined as the net effect of a single compound resulting from the simultaneous expression of two or more specific actions.

2 The principles of concentration-ratio analysis are extended to develop a method for detecting and quantifying competitive antagonism when this property is a component of a pharmacological resultant. The method is general to the extent that it allows analysis of competitive antagonism in combination with all types of post-receptor intervention. Essentially it depends on the altered expression of competition by a reference antagonist. It incorporates tests for validating its application and it is independent of agonist concentration-effect curve shape: in these respects the method is analogous to Schild plot-analysis of simple competition.

3 The methodology for the practical application of the analysis is exemplified by studying the net effect of a combination of a phosphodiesterase inhibitor (isobutylmethylxanthine) and histamine H_2-receptor antagonist (metiamide) on histamine-stimulated tachycardia in guinea-pig, isolated, right atrium. Cimetidine was used as the reference antagonist.

4 The equation used in this analysis is similar in form to one recently described by Hughes & Mackay (1985) to elucidate the situation when competitive antagonism occurs in combination with functional interactions. The relation between their method and the present analysis is discussed.

Introduction

In this paper pharmacological resultant is defined as the net effect of a single compound resulting from the simultaneous expression of two or more specific actions. Competitive antagonism expressed as part of a pharmacological resultant is now being recognized. Kenakin & Black (1978) showed that practolol was able to inhibit catechol *O*-methyl transferase (COMT) as well as antagonize β-receptors. This combination of properties led to a superimposition of leftward and rightward displacements of isoprenaline concentration-effect curves in rat heart muscle bioassays, resulting in under-expression of competitivity. Kenakin (1980) showed that metanephrine can inhibit extraneuronal catecholamine uptake as well as antagonize β-adrenoceptors leading to a similar self-cancelling pattern of agonist-concentration effect curve displacements. More recently, Kenakin & Beek (1985) demonstrated that ambenonium expresses both muscarinic receptor antagonism and acetylcholinesterase inhibition resulting in under expression of competitivity.

In each of these three cases the resultant problem could be resolved by choosing an agonist which is resistant to the disposition mechanism or by blocking that mechanism. Either way, the full expression of competitivity was then revealed. However, in principle not all examples of resultant actions can be resolved experimentally. Angus & Black (1980) argued that amitriptyline might express phosphodiesterase inhibition concomitantly with histamine H_2-receptor antagonism. This possibility was considered to account for the apparently low affinity expressed by amitriptyline for the histamine H_2-receptors in tissue bioassays compared with that expressed in adenylate cyclase assays (Kanof & Greengard, 1979). The known, leftward-shifting effect of phosphodiesterase

[1] Correspondence.

inhibition on histamine concentration-effect curves (Reinhardt *et al.*, 1977: Broadley & Wilson, 1980) would account for this discrepancy and indeed amitriptyline was later found to inhibit this enzyme (Reynolds & Claxton, 1982). In this case, there is no way of eliminating the phosphodiesterase component in tissue assays because inhibition of this enzyme produces full agonism. Another type of resultant effect which cannot be eliminated experimentally occurs when functional antagonism is combined with competitive antagonism, as was recently described for meptazinol, an opioid-receptor ligand (Goodall *et al.*, 1985).

The possibility of errors in the analysis of competitive antagonism caused by additional properties and the implications of these errors for receptor classification creates a need for a method by which resultant effects can be detected and the confounded competitive element estimated. In this paper we show how the principles of concentration-ratio analysis (Paton & Rang, 1965) may be extended to provide such a method. The theoretical basis for the method, which bears a close relation to Schild analysis of simple competition, is explained. Then, the method is exemplified practically, by making the assumption that a pair of substances each having a single dominant action can, when added together to the tissue bathing fluid in equivalent amounts, behave as though a single substance having both actions was present. The agents used were metiamide and isobutylmethylxanthine (IBMX), which in combination can be considered to produce a pharmacological resultant.

Recently, Hughes & Mackay (1985) arrived at a similar method by extending Mackay's (1981) treatment of functional interactions to include the additional property of competitive antagonism. The relation between their method and that presented here is discussed.

Theory

The analysis is based on the assumptions of the occupancy theory of agonist action. Pharmacological effect is assumed to be some monotonic function (F) of the concentration of agonist-occupied receptors. For the sake of simplicity, this function is expressed in terms of fractional occupancies (Stephenson, 1956). Thus:

$$E = F\left(\frac{[A]}{K_A + [A]}\right) \qquad (1)$$

in which [A] is the concentration of agonist and K_A is the dissociation constant for the agonist-receptor complex. This equation serves as a basis for establishing, initially, the conditions which permit analysis of simple competitive antagonism and, secondly, the ways in which additional properties invalidate these conditions.

Simple competitive antagonism

Simple competitive antagonism, by definition, can only effect the occupancy function. Receptor theory (Arunlakshana & Schild, 1959) predicts that a competitive antagonist, B, with dissociation constant K_B will affect Equation 1 as follows:

$$E^B = F\left(\frac{[A^B]}{\frac{K_A(1 + [B]) + [A^B]}{K_B}}\right) \qquad (2)$$

in which E^B signifies the effect and $[A^B]$ the concentration of A in the presence of B. For equal effects in the absence and presence of B,

$$E = E^B = F\left(\frac{[A]}{K_A + [A]}\right) = F\left(\frac{[A^B]}{\frac{K_A(1 + [B]) + [A^B]}{K_B}}\right) \qquad (3)$$

F, by definition, is unchanged by B, and so this function cancels allowing the following well-known relation (Arunlakshana & Schild, 1959):

$$r^B = \frac{[A^B]}{[A]} = 1 + \frac{[B]}{K_B} \qquad (4)$$

in which r^B defines the concentration-ratio of A required to overcome the competition by B. Measurement of r^B allows K^B to be estimated.

Resultant effects by competitive antagonists

In theory, there is a virtually unlimited number of ways in which a substance may interfere with the F ([A]) relation (Equation 1) independently of competition, by changing F in some way. In practice, all forms of post-receptor intervention and functional antagonism or synergism fall into this class of interaction. Where a ligand, C, acts to alter the transducer function F as well as compete with A, Equation 2 can be modified as follows:

$$E^C = F^C\left(\frac{[A^C]}{\frac{K_A(1 + [C]) + [A^C]}{K_C}}\right) \qquad (5)$$

Now, for equal effects in the absence and presence of C

(Equations 1 and 5), F and F^C cannot be cancelled. Therefore, the fundamental assumption which permits simple analysis of competition no longer holds.

Analysis of resultant effects using a standard competitive antagonist

Whenever a compound, C, is suspected of giving an effect which is the resultant of competitive antagonism plus some other action(s) then the unconfounded competitive element can be disclosed if a competitive antagonist B, which for practical purposes is free from significant resultant activity is available. The solution derives from the additive rule of Paton & Rang (1965), for two simple competitive antagonists acting at the same site. The agonist concentration-effect relation in the presence of both compounds, B and C, having dissociation constants K_B and K_C, becomes

$$E^{B+C} = F\left(\frac{[A^{B+C}]}{K_A\left(1 + \frac{[B]}{K_B} + \frac{[C]}{K_C}\right) + [A^{B+C}]}\right) \quad (6)$$

where E^{B+C} and $[A^{B+C}]$ signify the presence of both B and C. This equation represents the case when both B and C are simple competitive antagonists. However, when C expresses a resultant effect, that is when C also affects F, then Equation 6 must be modified accordingly:

$$E^{B+C} = F^C\left(\frac{[A^{B+C}]}{K_A\left(1 + \frac{[B]}{K_B} + \frac{[C]}{K_C}\right) + [A^{B+C}]}\right) \quad (7)$$

Although F^C still does not cancel if Equation 7 is compared with Equation 1 it does so when Equation 7 is compared with Equation 5 which represents the agonist concentration-effect relation in the presence of C alone. Thus for equal effects, equating (7) and (5),

$$E^C = E^{B+C} = F^C\left(\frac{[A^C]}{K_A\left(1 + \frac{[C]}{K_C}\right) + [A^C]}\right)$$

$$= F^C\left(\frac{[A^{B+C}]}{K_A\left(1 + \frac{[B]}{K_B} + \frac{[C]}{K_C}\right) + [A^{B+C}]}\right) \quad (8)$$

which, with the elimination of F^C and rearrangement gives,

$$r_C^{B+C} = \frac{[A^{B+C}]}{[A^C]} = 1 + \frac{[B]}{K_B\left(1 + \frac{[C]}{K_C}\right)} \quad (9)$$

where r_C^{B+C} defines the concentration ratio of A required to surmount the additional competition of B in the presence of C.

Therefore, for cases in which C expresses an effect resulting from both competitive antagonism and some additional action(s) on the transducer function F, then the competitive element in C, theoretically, can be estimated by measuring the additional concentration-ratio produced by a standard antagonist B, in the presence of C.

In the development of the model the competitive nature of the standard antagonist B, and the existence of a competitive action in the resultant profile of C was assumed. Although the credentials of the standard antagonist may be established independently, practical application of the model requires that Equation 9 be modified as follows to include the order terms n and m to provide criteria for simple competition in both the actions of B and C:

$$r_C^{B+C} - 1 = \frac{[B]^n}{K_B(1 + [C]^m/K_C)} \quad (10)$$

Practical estimation of the dissociation constant of a resultant competitive antagonist

Equation 10 can be written as

$$r_C^{B+C} = 1 + \frac{[B]^n}{K'_B} \quad (11)$$

where $K'_B = K_B(1 + [C]^m/K_C)$. In this form, equation 10 is seen to be the Schild equation in which K'_B is the apparent dissociation constant of B. In the presence of C, K_B is multiplied by the factor $(1 + [C]^m/K_C)$ and the ratio by which K'_B exceeds K_B allows the factor and therefore K_C to be estimated. Defining $K'_B/K_B = y$ then,

$$y - 1 = \frac{[C]^m}{K_C} \quad (12)$$

Ideally, y would be estimated at different values of [C]. A log $(y-1)$/log [C] plot would then provide an estimate of pK_C in an analogous way to that in which the conventional Schild plot analysis allows antagonist affinities to be estimated from displacements of agonist concentration-effect curves. Here, a series of Schild plots would be constructed as functions of [B], at different fixed values of [C], as illustrated in Figure 1. Thus, the displacements of these Schild lines, replotted according to Equation 12 allow estimation of the competitive component of C's resultant effect.

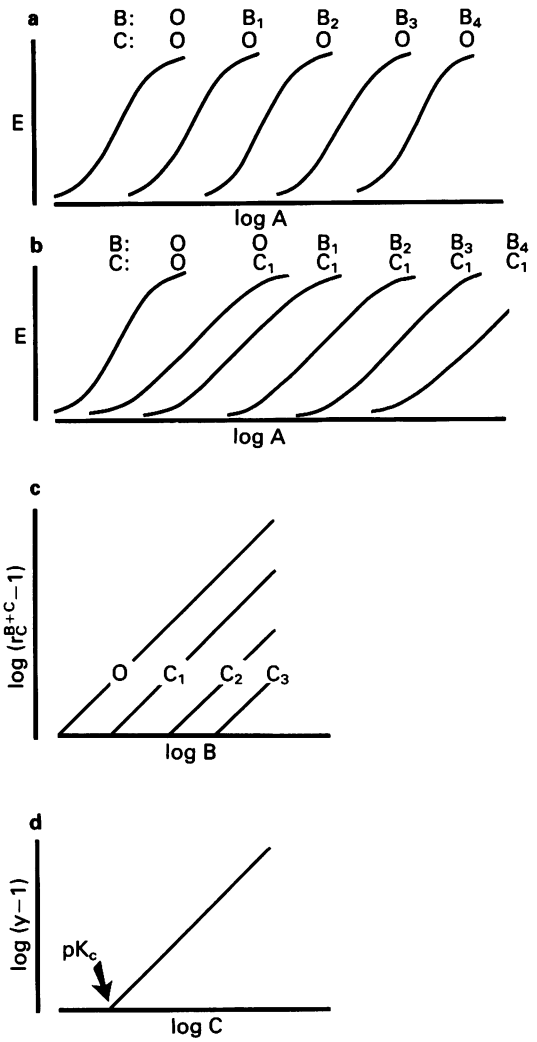

Figure 1 Principal steps in the concentration-ratio analysis. (a) Analysis of reference antagonist. The diagram illustrates the effects of four concentrations of B, $0-B_4$, on the agonist concentration-effect curve. (b) Analysis of B in the presence of test compound C at concentration C_1. Concentration-ratios elicited by B are measured between the curve obtained in the presence of C alone and the curve obtained in the presence of both C and B. (c) Repetition of (b) for different concentrations of C, C_1, C_2, C_3 to produce series of Schild lines. The distance between each displaced Schild line and the control plot on the log [B] axis is measured and defined as log (y). (d) Replot values for y for different concentrations of C, C_1, C_2, C_3. If C is competitive or has a competitive property amongst other independent properties then $y = 1 + [C]/K_C$ and a plot of log $(y-1)$/log [C] yields the value of the dissociation constant for C.

Methods

Guinea-pig, isolated, right atrium preparation

Chronotropic effects were recorded in the spontaneously beating right atrium preparation (Angus & Black, 1980) from male guinea-pigs (Halls, 350–425 g). The atria were suspended in an organ bath containing 20 ml of Krebs-Henseleit solution (composition mM: Na^+ 142.95, K^+ 5.87, Ca^{2+} 2.50, Mg^{2+} 1.18, Cl^- 127.64, $H_2PO_4^-$ 1.18, HCO_3^- 1.18 and dextrose 11.11) at 37°C gassed with 95% O_2 plus 5% CO_2.

The Krebs-Henseleit solution contained propranolol 10^{-7}M to eliminate the confounding influence of tissue release of catecholamines. After the initial resting tension was set at 500 mg the spontaneous rate was continuously recorded from a ratemeter.

Experimental protocols

Each preparation was allowed to stabilize for 60 min, during which time four changes of bath fluid were made. Then, the histamine H_2-receptor antagonists, metiamide and cimetidine and/or phosphodiesterase inhibitor, IBMX, were incubated for 1 h before obtaining full agonist concentration-effect curves using the selective H_2-agonist impromidine. Curves were constructed cumulatively at increments of 0.5 log units and each preparation was used once only.

The final volume of drug solutions added to the bath was not greater than 500 µl, i.e. 2.5% of bath volume. To minimize transient decreases in buffer pH resulting from additions of acidic impromidine trihydrochloride solution, stock concentrations were neutralized with NaOH. The excess Na^+ and Cl^- ions added as a result were shown independently to have no effect upon basal or stimulated activity (Black *et al.*, 1981).

Statistical methods

Impromidine concentration-effect curve data were fitted by means of an iterative least squares programme to a three-parameter logistic function of the form:

$$E = \frac{\alpha[A]^p}{[A_{50}]^p + [A]^p} \qquad (13)$$

in which α is the asymptote, $[A_{50}]$ is the value of [A] for 0.5α and p is the slope parameter. The programme also performed one-way analyses of variance for parallelism, comparing computed α and p values between and within antagonist concentration groups.

Analysis of simple competitive antagonism

For the analysis of simple competitive interactions, having made the above tests of parallelism, agonist concentration-effect curve data were fitted to the following equation (Stone & Angus, 1978):

$$E = \frac{\alpha[A]^p}{[A_{50}]^p (1 + [B]^n/K_B)^p + [A]^p} \quad (14)$$

in which $[A_{50}]$ is the concentration of A required for half maximal effect (0.5α) in the absence of antagonist; [B] is the antagonist concentration and K_B its dissociation constant at H_2-receptors; n is equivalent to the Schild plot slope parameter. If n was not significantly different from unity the data were refitted to Equation 14 with n constrained to unity and K_B was estimated directly in this fit.

Concentration-ratio analysis

The experimental results were analysed by fitting concentration-ratio data to the following equation:

$$r_C^{B+C} - 1 = \frac{[B]^n}{K_B (1 + [C]^m/K_C)} \quad (15)$$

where r_C^{B+C} is the concentration-ratio elicited by B, the reference competitive antagonist (cimetidine in these experiments), measured in the presence of C, the compound exhibiting resultant properties (represented by the IBMX-metiamide combination whose concentrations were varied in unison), from the position of the concentration-effect curves in the presence of B alone. K_C and K_B are the respective dissociation constants for C and B at the receptor (histamine-H_2 in this system). y represents the extent to which the apparent dissociation constant of B, K'_B, departs from its real value (K_B). In practice values of r_C^{B+C} were calculated at each concentration of C by dividing the computed mean $[A_{50}]$ value at [B] = 0 into individual $[A_{50}]$ values obtained at increasing values of [B].

Graphically, n and m correspond to the slopes of the log $(r_C^{B+C} - 1)$/log [B] and log $(y - 1)$/log [C] plots respectively. n and m must not be significantly different from unity for the antagonism of B and C, respectively, to conform to simple competition. As will be shown, neither criterion failed in practice. K_B and K_C were estimated from a subsequent fit of Equation 15 to concentration-ratio data with n and m constrained to unity.

Compounds

Isobutylmethylxanthine (Sigma), d(±)-propranolol hydrochloride (Sigma), impromidine trihydrochloride (Wellcome Research Laboratories), cimetidine (Smith Kline and French Laboratories) and metiamide (gift from Dr M. Parsons of Smith Kline and French Laboratories).

Results

Figures 2 and 3 show the results of experiments

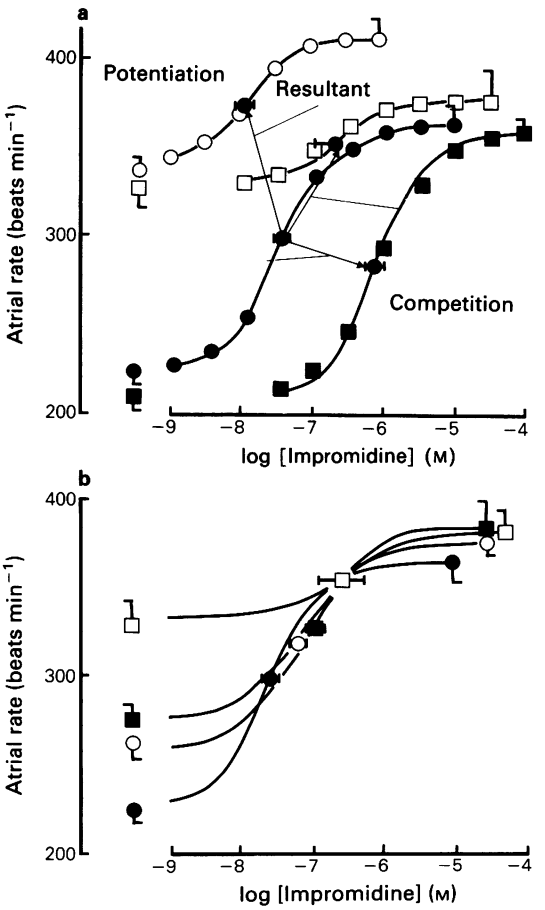

Figure 2 The effect of isobutylmethylxanthine (IBMX)-metiamide on impromidine concentration-effect curves. (a) Shows the average logistic-fitted impromidine curves superimposed on experimental data points in the absence (●, $n = 15$) and presence of IBMX (3×10^{-5}M; ○, $n = 5$), metiamide (3×10^{-5}M, ■, $n = 5$) and IBMX-metiamide (3×10^{-5}M; □, $n = 6$). (b) Average logistic-fitted impromidine curves in the absence (●, $n = 15$) and presence of 3×10^{-6} (○, $n = 6$), 10^{-5} (■, $n = 6$) and 3×10^{-5}M (□, $n = 6$) IBMX-metiamide. For clarity, only computed basal, upper asymptote and log $[A_{50}]$ values are shown together with standard errors.

designed to simulate a pharmacological resultant and its elucidation by application of combined concentration-ratio analysis. The equimolar combination of IBMX and metiamide was meant to represent the potential of a compound, perhaps amitriptyline, to exhibit the pharmacological resultant of H_2-receptor blockade plus phosphodiesterase inhibition (Angus & Black, 1980). Figure 2b shows the effects of this combination on impromidine concentration-effect curves. Four concentrations of the IBMX-metiamide mixture was used: zero: 3×10^{-6}M: 10^{-5}M: 3×10^{-5}M. Figure 2a represents the effects, measured in separate experiments, of IBMX and metiamide alone, each at 3×10^{-5}M. Comparison with the combined effects at this concentration illustrates the degree of mutual camouflage by the two activities.

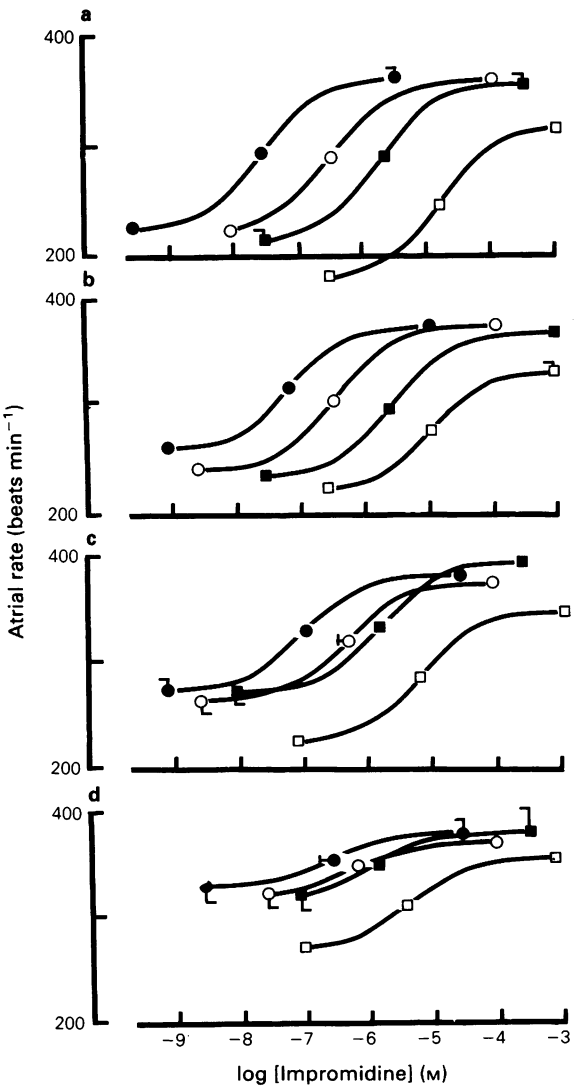

Figure 3 shows the interaction between cimetidine and each IBMX-metiamide combination. At each concentration of the combination, cimetidine was also applied at concentrations ranging from zero to 6×10^{-4}M. Logistic fitting and associated tests (see Methods) indicated that the sets of curves at zero, 3×10^{-6}M and 10^{-5}M IBMX-metiamide were parallel. As a three-parameter logistic function was used, data fitting and tests for parallelism only referred to responses developed above threshold: the absolute vertical location of the curves was not taken into account. At 3×10^{-5}M IBMX-metiamide, significant non-parallelism was detected and an amplitude change was also observed which was attributable to the increased amplitude of the agonist concentration-effect curve at 6×10^{-4}M cimetidine compared with those at low concentrations of cimetidine. A t test comparing control threshold responses values to those in the presence of cimetidine revealed significant depression by 6×10^{-4}M cimetidine ($P < 0.05$) in all treatment combinations which caused the vertical downward displacement, without alteration of curve amplitude, at IBMX-metiamide concentrations zero to 10^{-5}M. Lower concentrations of cimetidine did not produce significant depressions at any of the concentrations of IBMX-metiamide. These effects of cimetidine were, in part at least, clearly independent of the H_2-receptor antagonism and are discussed later.

Concentration-ratio data were obtained from the midpoints of individually-fitted curves as described in the statistical section and this information is displayed in Figure 4 in Schild-plot form. Fitting of all the concentration-ratio data to Equation 15 indicated that n and m were not significantly different from unity ($n = 0.92 \pm 0.08$ (s.e.): $m = 1.16 \pm 0.33$). Hence n and

Figure 3 Effect of variable cimetidine on impromidine concentration-effect curves at different fixed concentrations of isobutylmethylxanthine (IBMX)-metiamide. Replicate impromidine-effect curves were obtained in the absence (a) and presence of IBMX-metiamide 3×10^{-6}M (b), 10^{-5}M (c), 3×10^{-5}M (d), and the following molar concentrations of cimetidine: (a) 0.0 (●), 6×10^{-6} (○), 6×10^{-5} (■) and 6×10^{-4} (□). (b) 0.0 (●), 10^{-5} (○), 7.5×10^{-5} (■), 6×10^{-4} (□). (c) 0.0 (●), 2×10^{-5} (○), 10^{-4} (■), 6×10^{-4} (□). (d) 0.0 (●), 4×10^{-5} (○), 1.5×10^{-4} (■), 6×10^{-4} (□).

The line shown for each combination of agents represents the average logistic curve for that group of replicate curves. Average threshold and maximal asymptotes were calculated from the experimental data and midpoints of curves are the geometrically averaged values of the computed $[A_{50}]$ values for each group. Standard error bars are shown with the mean values when they are not contained within the graphical symbols.

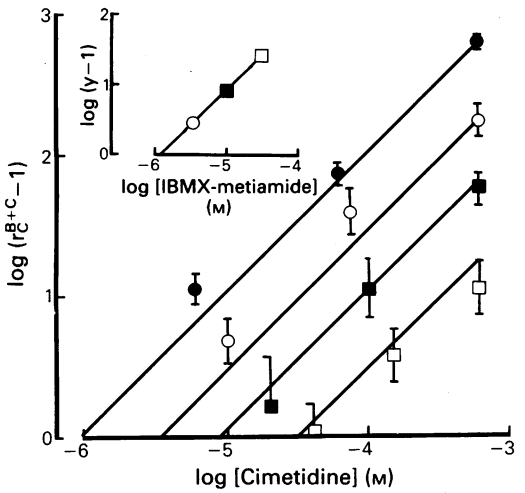

Figure 4 Schild plots with cimetidine as variable antagonists in the absence (●) and presence of different fixed concentrations of isobutylmethylxanthine (IBMX)-metiamide, (○) 3×10^{-6}M, (■) 10^{-5}M and (□) 3×10^{-5}M. Values of ($r_C^{B+C}-1$) were calculated as described in the theoretical and statistical sections. The average concentration-ratio values are shown with 95% confidence intervals. The slope parameters for the log ($r_C^{B+C}-1$)/log [B] plot and the log (y − 1)/log [C] plot were both not significantly different from unity (see text). The log (y − 1)/log [C] plot corresponding to the fit is shown inset. The calculated value of K_C was $1.14 \pm 0.18 \times 10^{-6}$M (s.e.) which is equivalent to a pK_C value of 5.94.

m were constrained to unity in a subsequent fit to Equation 15, giving computed values of the dissociation constants as follows: $K_B = 9.54 \pm 0.21 \times 10^{-7}$M (s.e.): $K_C = 1.14 \pm 0.18 \times 10^{-6}$M. The inset shows the log (y − 1) versus log [C] plot equivalent to the fit.

K_B ($8.78 \pm 1.11 \times 10^{-7}$M (s.e.)) could be estimated independently from the data shown in Figures 3 and 4 at zero IBMX-metiamide using Equation 14. The calculation of K_B from the concentration-ratio data is a product of an analysis particularly designed to estimate K_C (the unknown dissociation constant), and it is essential that K_B is correctly estimated. Comparison of the two estimates of K_B indicates that this was the case.

In order to test the ability of the concentration-ratio analysis to quantify the obscured antagonism accurately, metiamide was analysed independently in a simple competitive study, using the following concentrations (with a number of replicates in parentheses): zero M (23): 3×10^{-6}M (6): 6×10^{-6}M (6): 10^{-5}M (6); 3×10^{-5}M (6); 6×10^{-5}M (6); 10^{-4}M (6): 6×10^{-4}M (6). Metiamide produced parallel displacements of the impromidine concentration-effect curve over 2.5 orders of magnitude. Analysis using Equation 14 gave an estimate of n of 1.04 ± 0.04 (s.e.). With n constrained to unity the estimated equilibrium dissociation constant (equivalent to K_C in the previous section) was $1.21 \pm 0.49 \times 10^{-6}$M (s.e.).

Discussion

Concentration-ratio analysis is an established pharmacological technique for the analysis of interactions between antagonists. Paton & Rang (1965) showed that when two antagonists are mutually competitive as well as being competitive with the agonist, the concentration-ratios combine additively. When two antagonists act at independent sites the concentration-ratios combine in a multiplicative way. Essentially, the interaction between a competitive and non-competitive antagonist falls into the latter multiplying group of interactions. While the method of Paton & Rang (1965) distinguished between competitive and such 'non-competitive' interactions it does not cater for circumstances where both properties occur in combination in a single chemical entity. This study shows how the principle of concentration-ratio analysis may be extended to enable detection and quantification of competition when it occurs in such a 'resultant' situation.

Using the combination of a competitive antagonist and a potentiating compound the experimental study was undertaken to illustrate the principle of the analysis. The method appeared to be successful as judged by the estimate of metiamide's affinity at H_2-receptors when its expression was camouflaged by concurrent phosphodiesterase inhibition. This model study thus establishes the potential value of the method as a tool during pharmacological classification studies of substances which express multiple, interacting properties. The possibility of a resultant pharmacological effect entails that some novel agents may well be concluded to be 'inactive' when, in fact, competition is present but camouflaged by some additional potentiating property. Alternatively, competition may be overestimated due to an additional 'right-shifting' property.

The criteria for the detection and quantification of confounded competition by this method have clear parallels in the 'Schild plot' method of analysis (Arunlakshana & Schild, 1959). In order to estimate the dissociation constant of a simple competitive antagonist a reference agonist is required. In the present analysis, a reference competitive antagonist must be available. Cimetidine was chosen for use in these studies because it is a generally accepted, well-classified competitive antagonist of histamine H_2-receptors (Brimblecombe *et al.*, 1975: Durant *et al.*, 1977). In fact, results here showed that cimetidine

produced a depressant effect at the highest concentration used in the absence and presence of the test combination, IBMX-metiamide. Thus, strictly, cimetidine fails one of the criteria of simple competition (Schild, 1973; Black *et al.*, 1982). However, analysis of the horizontal location changes of agonist concentration-effect curves induced by cimetidine indicated that the depressant property did not interfere with the expression of its competition, as judged by adherence to Equation 14 in which n was found not to be significantly different from unity. This test is, of course, merely corroborative of the assumption of cimetidine's competitive property based on anterior evidence (eg Brimblecombe *et al.*, 1975; Black *et al.*, 1985).

A much more important test is the evaluation of parameter m in Equation 15 which tests the competitive nature of the antagonist element in the resultant 'mixture'. As in a first order Schild analysis, significant departure from linearity provides positive grounds for rejecting the simple competitive hypothesis while absence of significant non-linearity provides necessary but not sufficient grounds for competition. In this case, m was not significantly different from unity and so competition would not be rejected, probably correctly because of the known properties of metiamide (eg Black *et al.*, 1973). Obviously, while confidence in the combined concentration-ratio method is justified when an ideally behaved reference antagonist is available, it is apparent that the method suffers from the same problems of circularity frequently encountered in conventional pharmacological classification studies.

The lower order version of Equation 15 which was recently described by Hughes & Mackay (1985) provides no tests for the nature of the antagonism present in test or reference compounds. Hughes & Mackay considered that this equation should 'provide information about the value K_{I1} (equivalent to K_C in the present analysis) in all circumstances'. However, while prior tests for the nature of the antagonism expressed by the reference compound will be available beforehand, prior information about the competitive nature (or even the existence) of this antagonism by the test compound cannot be so obtained. In their method there is no way of knowing that the effect of the test compound on the expression of competition by the reference compound is due to a competitive antagonist action. In the present method the determination of the parameter m (Equation 15), provides a necessary but not sufficient test for competition.

Clearly, further practical exemplification of this combined concentration-ratio method is required. As in the case of the Schild analysis of simple competition, although the method is theoretically valid, its practical utility can only be established with repeated application. The method seems to us to represent a useful addition to existing pharmacological classification of compounds exhibiting multiple properties, problems which cannot be approached by existing techniques.

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