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Application of an Allosteric Ternary Complex Model to the Technique of Pharmacological Resultant Analysis

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Abstract

A simple ternary complex model of drug-receptor interaction has been used to extend the procedure of pharmacological resultant analysis, enabling the quantitation of interactions between allosteric modulators and orthosteric antagonists.

Equations derived in the theoretical treatment were used to analyse functional data for the interaction between the allosteric modulator gallamine and the orthosteric antagonist scopolamine, with oxotremorine as the agonist, at rat tracheal muscarinic acetylcholine receptors. Quantitative estimates of the affinity of gallamine for the allosteric site ($pK_Z = 4{\cdot}7$) and the extent of negative, heterotropic co-operativity between gallamine and scopolamine ($\alpha' = 13{\cdot}1$) were obtained. Furthermore, an alternative direct, model-fitting approach, that does not rely on the determination of concentration ratios, was also developed, and yielded similar results.

It is suggested that the approach presented in this paper is useful for quantifying interactions between orthosteric antagonists and allosteric modulators, particularly when the extent of co-operativity is low or the modulators possess multiple pharmacological properties, or both.

Functional experiments have long relied on null-methods for the quantitation of biologically relevant drug-receptor parameters, such as agonist and antagonist dissociation constants, and agonist relative efficacies (Kenakin 1993). Many of these null-methods, in essence, involve the distillation of agonist concentration-response curves to a series of concentration ratios, on the basis of location parameters that represent equiactive response levels, before the application of the desired analytical approach (Arunlakshana & Schild 1959).

Paton & Rang (1965) demonstrated how concentration ratios derived in the absence and presence of combinations of antagonists could be assessed in terms of both competitive and non-competitive drug-receptor models, of which allosteric interactions are an example (Stockton et al 1983; Ehlert 1988). The technique of concentration ratio analysis, based on the method of Paton & Rang (1965), has been modified and applied to the interaction of allosteric modulators with agonists and traditional orthosteric antagonists, at muscarinic acetylcholine receptors (Mitchelson 1975; Christopoulos & Mitchelson, 1994). These analyses have been used to derive estimates of co-operativity factors (α') for the interaction between the bisquaternary allosteric modulator heptane-1,7-bis-(dimethyl-3′-phthalimidopropyl)ammonium bromide and orthosteric antagonists such as atropine, dexetimide and *N*-methylscopolamine, at muscarinic receptors.

Black et al (1986) have also extended and utilized the principles of concentration-ratio analysis to quantify competitive antagonism that occurs as part of a pharmacological resultant, i.e. a net effect produced by a single compound which results from the simultaneous expression of more than one specific action. This method of pharmacological "resultant analysis" has been successfully employed, for example, to dissect the purely syntopic components of the actions of cimetidine in guinea-pig ventricles (Trist et al 1987) and of theophylline and isobutylmethylxanthine in rat vas deferens and guinea-pig atria (Kenakin & Beek 1987). A similar type of analysis has been derived and utilized by Hughes & Mackay (1985) and Goodall et al (1985) to study the actions of mixed competitive and functional antagonists. Theoretically, these methods offer advantages over traditional concentration ratio analyses (Paton & Rang 1965; Kenakin 1993).

There is no reason to assume that allosteric modulators might not also express other pharmacological properties. Indeed, the anticholinesterase actions of the modulator gallamine at high concentrations, when using acetylcholine as the agonist at muscarinic receptors (Roufogalis & Quist 1972; Clark & Mitchelson 1976), testify to such a phenomenon. However, as initially proposed, the model underpinning the technique of resultant analysis is inappropriate for quantifying the allosteric effects of such compounds. We present a theoretical development which modifies the model of Black et al (1986) to accommodate an allosteric modulator. Furthermore, this same approach can be used as an alternative method to that presented previously (Christopoulos & Mitchelson 1994) for estimating the co-operativity factor (α') for the interaction between an allosteric modulator and an orthosteric antagonist in functional experiments, and this should be detectable irrespective of whether or not the modulator possesses additional pharmacological properties.

Theory

If the pharmacological effect, E, is assumed to be a monotonic function, f, of receptor occupancy, and the agonist, A, binds in a non-co-operative manner, then:

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

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where [A] is the concentration of agonist and K_A is the agonist-receptor equilibrium dissociation constant. For simple, competitive (orthosteric) antagonism (Scheme 1A) where only occupancy and not f is affected:

$$E_B = f\left(\frac{[A]_B}{[A]_B + K_A(1 + [B]/K_B)}\right) \quad (2)$$

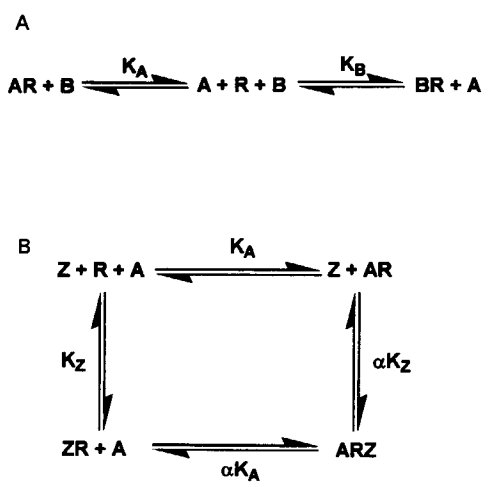
where [B] denotes the concentration of antagonist, K_B the antagonist-receptor equilibrium dissociation constant, and E_B and $[A]_B$ denote the effect and the concentration of agonist, respectively, in the presence of antagonist B.

The simplest scheme of allosteric interaction at G protein-coupled receptors involves the formation of a ternary complex between an orthosteric ligand, A, an allosteric modulator, Z, and the receptor, R (Scheme 1B). For allosteric inhibition by Z, where only agonist occupancy is affected, the following relationship can be derived (Ehlert 1988):

$$E_Z = f\left(\frac{[A]_Z}{[A]_Z + K_A\left(\frac{[Z] + K_Z}{[Z]/\alpha + K_Z}\right)}\right) \quad (3a)$$

where [Z] denotes the concentration of modulator, K_Z the modulator-receptor equilibrium dissociation constant, α the co-operativity factor for the interaction between the agonist and the modulator, and E_Z and $[A]_Z$ denote the effect and the concentration of agonist, respectively, in the presence of modulator Z.

For equal effects, E from equation 1 can be equated with either E_B from equation 2, for competitive antagonism, or E_Z from equation 3a, for allosteric inhibition, and the function f cancels, enabling quantitative analysis of the interaction between A and B, or between A and Z, to be undertaken using standard null-methods (Arunlakshana & Schild 1959; Ehlert 1988).



Scheme 1. A. Model of simple, competitive antagonism between two orthosteric (syntopic) ligands, agonist A and antagonist B, interacting at a shared binding site on the receptor, R, according to the law of mass action. AR and BR denote the drug-receptor complexes whereas K_A and K_B denote their respective equilibrium dissociation constants. B. Ternary complex model of allosteric interaction. R denotes the receptor, A and Z denote the orthosteric and allosteric ligands, respectively, K_A and K_Z denote the equilibrium dissociation constants of AR and ZR, respectively. The symbol α denotes the co-operativity factor, a quantitative measure of the maximum reciprocal alteration of affinity of A and Z for their respective binding sites, when both ligands bind concomitantly to form the ternary complex ARZ.

However, if the allosteric modulator possesses another property which in some way alters the stimulus-response function, then equation 3a can be expressed as:

$$E_Z = f_Z\left(\frac{[A]_Z}{[A]_Z + K_A\left(\frac{[Z] + K_Z}{[Z]/\alpha + K_Z}\right)}\right) \quad (3b)$$

where f_Z denotes the altered stimulus-response function. A comparison of equation 1 with equation 3b shows that, in this instance, f and f_Z do not cancel, and standard null-methods cannot be readily employed. Ehlert (1988) has discussed this problem and indicated certain situations where quantitative analysis of modulator-agonist interactions can be undertaken. However, as shown previously (Christopoulos & Mitchelson 1994), when Z and A are combined with an orthosteric antagonist (reference antagonist), B, which is known not to affect f, then the following expression can be derived:

$$E_{BZ} = f_z\left(\frac{[A]_{BZ}}{[A]_{BZ} + \frac{\alpha K_A K_Z}{\alpha K_Z + [Z]}\left(1 + [B]/K_B + [Z]/K_Z + \frac{[B][Z]}{\alpha' K_B K_Z}\right)}\right) \quad (4)$$

where E_{BZ} and $[A]_{BZ}$ denote the effect and concentration, respectively, of the agonist in the presence of both B and Z, and α' denotes the negative heterotropic co-operativity factor for the interaction between B and Z.

Let $K'_A = K_A\left(\frac{[Z] + K_Z}{[Z]/\alpha + K_Z}\right)$ and

$$K_{A''} = \frac{\alpha K_A K_Z}{\alpha K_Z + [Z]}\left(1 + [B]/K_B + [Z]/K_Z + \frac{[B][Z]}{\alpha' K_B K_Z}\right)$$

then, for equal effects:

$$E_Z = E_{BZ} = f_Z\left(\frac{[A]_Z}{[A]_Z + K_{A'}}\right) = f_Z\left(\frac{[A]_{BZ}}{[A]_{BZ} + K_{A''}}\right) \quad (5)$$

Hence, in this instance, f_Z can be cancelled and the resulting expression can be simplified and rearranged to:

$$\frac{[A]_{BZ}}{[A]_Z} = \frac{K_{A''}}{K_{A'}} = CR_Z^{BZ} \quad (6)$$

where CR_Z^{BZ} denotes the concentration ratio of A, combined with Z, in the absence and presence of B.

Substituting for $K_{A'}$ and K''_A and simplifying yields:

$$CR_Z^{BZ} = 1 + \frac{[B]}{K_B}\frac{\alpha' K_Z + [Z]}{\alpha'(K_Z + [Z])} \quad (7)$$

$$\text{If } K_{B'} = \frac{K_B \alpha'(K_Z + [Z])}{\alpha' K_Z + [Z]}, \text{ then :} \quad CR_Z^{BZ} - 1 = \frac{[B]}{K_{B'}} \quad (8a)$$

An order term, s, can be included in the above equation for practical application of the model, thus providing criteria for ascertaining simple, orthosteric antagonism by B:

$$CR_Z^{BZ} - 1 = \frac{[B]^s}{K_{B'}} \quad (8b)$$

Thus, in the presence of Z, K_B is multiplied by $\frac{\alpha'(K_Z + [Z])}{\alpha' K_Z + [Z]}$, a factor which can be determined from the ratio by which $K_{B'}$ exceeds K_B.

If $K_{B'}/K_B$ is defined as Y, then:

$$Y = \frac{\alpha'(K_Z + [Z])}{\alpha' K_Z + [Z]} \quad (9)$$

and

$$Y - 1 = \frac{[Z](\alpha' - 1)}{\alpha' K_Z + [Z]} \quad (10)$$

which is similar to the equation relating the concentration ratio of an agonist in the absence and presence of allosteric modulator, Z, as derived by Ehlert (1988). Hence, a plot of log (Y − 1) against log [Z] will yield a curve with the x-intercept corresponding to log K_Z and the asymptote yielding α', the negative, heterotropic co-operativity factor for the interaction between an orthosteric antagonist and an allosteric modulator.

Recently, Lew & Angus (1995) described an alternative approach for analysing competitive interactions that by-passed the need to calculate concentration ratios. Instead, the concentration–response curve EC50 values were directly fitted to a model of competitive interaction using non-linear regression. This approach offers theoretical advantages over traditional ratio-based methods. In a similar fashion, the concentration ratio-based approach described above can also be replaced with an alternative location-parameter-based approach. In this instance, however, the parameter of interest is taken from the orthosteric antagonist Schild regressions, in the absence and presence of the various concentrations of modulator. Thus, equation 9 can be re-arranged to solve for $K_{B'}$. Taking negative logarithms then yields an expression for $pK_{B'}$, the negative x-intercept of each orthosteric-antagonist Schild regression, constrained to unity. Subsequently, the control pK_B and all other $pK_{B'}$ values can be fitted to the equation:

$$pK_{B'} = -\log.([Z] + 10^{-pK_Z}) + \log.([Z]/\alpha' + 10^{-pK_Z}) - \log.d \quad (11)$$

where d is the product of α' and [B], in the absence of [Z]. The relevant parameters can then be derived by non-linear regression analysis of a plot of $pK_{B'}$ against [Z], on a linear scale. As this method includes the control pK_B (i.e. in the absence of modulator) in the analysis, an additional degree of freedom is gained.

Methods

In the following discussion, it is assumed that equilibrium conditions have been experimentally established throughout. In practice, the technique initially requires the establishment of a simple, competitive mode of interaction for a reference, orthosteric, antagonist. This can be performed by Schild analysis, although it should be stressed that the method of Black et al (1986) contains an internal check of this null hypothesis. Subsequently, control agonist concentration-response curves are established in the presence of a fixed concentration of the compound of interest (the modulator, or test antagonist), the tissue is washed free from drugs, and the same concentration of test antagonist is re-added, together with a fixed concentration of reference antagonist. The concentration-response curve to the agonist is then re-established, resulting in dextral displacement of the control concentration-response curve. The ratio of locations of the control and shifted curves, assuming the displacement is parallel with no change in maximum agonist effect, can then be utilized in the derivation of CR_Z^{BZ}, as defined in the Theory section.

A series of experiments is performed in this manner, utilizing different concentrations of reference and test antagonists. The result is a series of reference antagonist Schild regressions which are progressively shifted to the right as the concentration of test antagonist is increased. These shifts are used to derive the resultant plot for the interaction between reference and test antagonist. The quantitative aspects of the method outlined above were evaluated by using the published data of Kenakin & Boselli (1989) for the interaction of the test antagonist gallamine, and the reference antagonist scopolamine at muscarinic receptors in the rat trachea, with oxotremorine as the agonist.

Results

The data of Kenakin & Boselli (1989) were used to test the allosteric model of resultant analysis derived above. In their study, the authors measured the gallamine- and pirenzepine-induced dextral displacements of the Schild regressions for scopolamine antagonism of rat-tracheal responses to a number of muscarinic agonists.

When gallamine was tested against scopolamine, with oxotremorine as the agonist, the resultant displacements of the scopolamine Schild regression provided four points for the resultant plot (Kenakin & Boselli 1989). Other experiments with gallamine or pirenzepine, tested against scopolamine, yielded resultant regressions with either two or three points. Thus, the current analysis utilized the gallamine-scopolamine-oxotremorine data.

Fig. 1 shows the re-plotted gallamine-scopolamine resultant data. A linear regression through the data points yielded a slope of 0·6, identical with that derived by the original authors. Constraining the slope to unity yielded a value for the negative logarithm of the x-axis of 4·3, again identical to the original authors' estimate. Hence, it may be concluded that reading the published data by eye did not appear to significantly distort the results. Statistical analysis of these parameters was not attempted, as the original authors claimed the data-point numbers were insufficient to yield reasonable error estimates. A regression through the data points according to an allosteric model (equation 10) is also shown in Fig. 1.

As the experimental design, with regard to performing resultant analysis, often precludes the construction of more than 2–3 concentration-response curves per tissue per day, data generated from such experiments might, theoretically, be more amenable to a global, location-parameter-based method of analysis, because the control Schild regression, in the absence of modulator, might also be incorporated into the fit. Accordingly, Fig. 2 shows the same data analysed according to

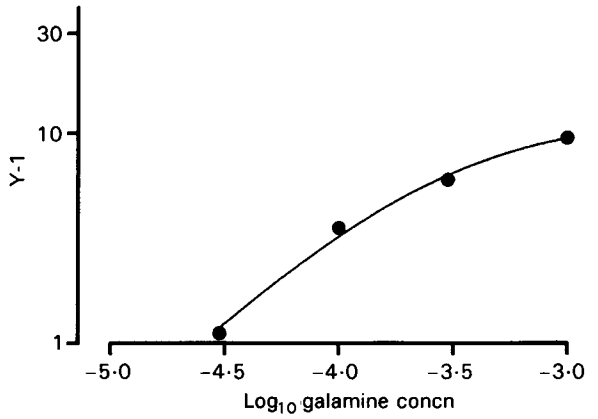


Fig. 1. Resultant regression of the gallamine–scopolamine interaction, with oxotremorine as the agonist, on the basis of the data of Kenakin and Boselli (1989). The negative logarithm of the equilibrium dissociation constant for gallamine at the allosteric site ($pK_Z = 4{\cdot}7 \pm 0{\cdot}3$) and the co-operativity factor for the interaction between gallamine and scopolamine ($\alpha' = 13{\cdot}1 \pm 1{\cdot}3$), were derived by fitting the data according to an allosteric model (equation 10). $Y - 1$ is the ratio of scopolamine Schild regression x-axis intercepts, in the presence and absence of gallamine, minus one.

equation 11. It is apparent that, in this instance, estimates of pK_Z and α' are almost identical from both analyses. It should also be noted that the standard error estimates given in Figs 1 and 2 are from the current, non-linear regression analyses, according to the allosteric model.

Discussion

The concomitant existence of both an inhibitory effect on tissue responses to acetylcholine at muscarinic receptors and an anti-cholinesterase property in the actions of gallamine (Clark & Mitchelson 1976) is an example of a pharmacological resultant (Black et al 1986). Examples of competitive antagonism as part of such a resultant have been recognized for some time (Black et al 1986). In some instances the problem of dissecting and quantifying the competitive component present in the resultant effect can be circumvented. For example, Clark & Mitchelson (1976) used the irreversible anticholinesterase, dyflos, to unmask and cancel the obscuring effects that the anticholinesterase properties of gallamine exerted on the gallamine-acetylcholine Schild regression. However, such avenues might not always be available, and methods have been developed which extend the principles of concentration-ratio analysis to identify and quantify competitive antagonism as part of a pharmacological resultant (Hughes & Mackay 1985; Black et al 1986). Application of this technique by Kenakin & Boselli (1989) to the study of the actions of gallamine and pirenzepine in rat trachea led the authors to conclude that previous reports of non-competitive behaviour with these agents (Clark & Mitchelson 1976; Choo et al 1985), based on Schild analyses, were not because of a pharmacological resultant, but were, in fact, because of the interaction of these ligands with an allosteric site.

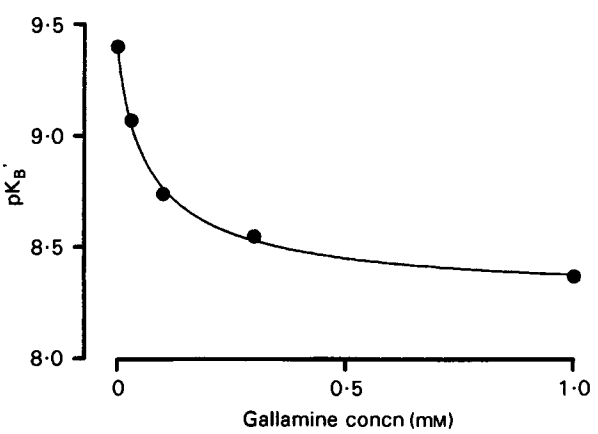


Fig. 2. A global, location-parameter-based regression analysis of the same experiments as for Fig. 1. Data were fitted to equation 11. Estimates of pKz and α' were $13{\cdot}2 \pm 1{\cdot}3$ and $4{\cdot}7 \pm 0{\cdot}2$, respectively. Note, however, the extra data point compared with the analysis in Fig. 1. pK_B' denotes the scopolamine Schild regression x-axis intercepts in the absence and presence of various concentrations of gallamine, taken from Kenakin and Boselli (1989).

In the model of Black et al (1986), the following equation provides a presumptive check for orthosteric antagonism in the actions of both a reference antagonist, B, and a test antagonist, C:

$$CR_C^{BC} - 1 = \frac{[B]^s}{K_B(1 + [C]^m/K_C)}$$

where CR_C^{BC} defines the concentration ratio of the agonist required to overcome the additional competition of B in the presence of C. The terms s and m are order terms, and should not be significantly different from unity for simple, competitive antagonism. The model thus contains an internal check for simple competitive antagonism between B and C. In an earlier study, Kenakin & Beek (1987) outlined three main criteria that must be satisfied for proper application of the above equation. Firstly, the reference antagonist must produce parallel, dextral shifts of the agonist concentration-response curves, in the absence and presence of the test antagonist. Secondly, the dextral displacements of the Schild regressions of the reference antagonist, in the presence of increasing concentrations of the test antagonist, must also be parallel. Thirdly, the resultant plot of these dextral Schild displacements must yield a regression with a slope not significantly different from unity; this constrains the value of m to unity in the above equation. In the study of Kenakin & Boselli (1989), the first two criteria were met, but the third was not. This was taken as additional, and more definitive, evidence, to that provided by Schild analysis, that these compounds did not appear to recognize the orthosteric site.

It should be noted that, in essence, the conclusion of a truly non-competitive mode of interaction for gallamine was adequately determined by the application of resultant analysis in its original form. This is so because the discriminatory capabilities of the technique rely on the practical methodology employed. The quantitative capabilities, however, rely on the underlying model and accompanying assumptions. Accordingly, the current study has modified the model of Black et al (1986) to enable quantitation of allosterism. Equation 8b can be rewritten:

$$CR_Z^{BZ} - 1 = \frac{[B]^s}{K_B} \cdot \frac{\alpha' K_Z + [Z]}{\alpha'(K_Z + [Z])}$$

A comparison with the preceding equation of Black et al (1986) reveals the necessary modifications required for the

quantitation of allosteric phenomena under these conditions. If necessary, an order term can be introduced for co-operative binding of [Z], with respect to itself, although the current formulation of the model does not make this assumption.

The practical requirement for a reference, orthosteric antagonist for performing resultant analysis assigns an additional theoretical utility to this technique, namely, the functional quantitation of allosteric interactions between any orthosteric antagonist-allosteric modulator pair. The presence or absence of a pharmacological resultant in the actions of the modulator is irrelevant in this instance. Thus, the method presented here offers an alternative means for the estimation of α' to the method presented earlier by Christopoulos & Mitchelson (1994). Furthermore, this method of analysis enables estimation of the dissociation constant of the modulator as a fitted parameter. In contrast, the earlier method of Christopoulos & Mitchelson (1994) required the separate estimation of this parameter, before determination of α'.

Application of equation 10 to the data of Kenakin & Boselli (1989) yielded estimates of pK_Z and α' for the gallamine-scopolamine interaction in the rat trachea of 4·7 and 13·1, respectively. An alternative, but related, approach to this type of analysis is a method that does not rely on ratios, but uses, instead, the absolute values of the pK_B and $pK_{B'}$ estimates from the Schild regressions of the reference antagonist. This method is an example of a global, location-parameter-based, non-linear regression technique, as suggested by Lew & Angus (1995); application of equation 11 to the data yielded a pK_Z of 4·7 and an α' of 13·2. Thus, excellent agreement was obtained between the two methods.

Ehlert (1988) has shown that the pA_2 value of a Schild regression for an agonist-modulator pair can often furnish a good estimate of true pK_Z, if α is greater than ca 10. Kenakin & Boselli (1989) found a pA_2 value of 4·7 for the gallamine-oxotremorine interaction, the same as that estimated from the gallamine-scopolamine resultant regression, on the basis of an allosteric model. This would be expected if gallamine were interacting with the same accessory binding site on tracheal muscarinic receptors to exert its modulatory effects on both agonists and antagonists. On the other hand, linear regression of the resultant data, as required for the application of resultant analysis of competitive interactions, gave a poorer fit with an apparent pK_Z of 4·3. Nevertheless, values of 4·3–4·7 are in the range of pA_2 estimates for gallamine at M_3 muscarinic receptors, the receptors believed to be the major mediators of tracheal smooth muscle contraction in response to acetylcholine (Roffel et al 1988; Michel et al 1990).

Although no other studies have investigated the allosteric interaction between gallamine and scopolamine at M_3 receptors, binding studies at cloned M_3 receptors have indicated high amounts of negative co-operativity, i.e. $\alpha' > 72$, between gallamine and [^{3}H]*N*-methylscopolamine (Lee & El-Fakahany 1991), a structurally-related antagonist. This value is markedly higher than the low extent of co-operativity reported in the current study. These differences might be attributed to the presence of the methyl group in *N*-methylscopolamine, compared with scopolamine, or to the different experimental conditions used by Kenakin & Boselli (1989) and Lee & El-Fakahany (1991). Furthermore, isolated-tissue experiments conducted by Choo (1987), yielded similar results to those reported by Kenakin & Boselli (1989), indicating a non-competitive interaction between gallamine and oxotremorine in ileal smooth muscle.

The main disadvantage of this approach is in terms of its overall practical application. Combinations of various concentrations of inhibitors (orthosteric or allosteric, or both) limit the agonist concentration ranges over which concentration-response curves might be reasonably displaced. This can lead to the generation of insufficient data points on the resultant plot for efficient statistical analysis of generated parameter estimates. Nevertheless, it is suggested that this technique is suitable for ligands interacting with low negative co-operativity. In such instances, agonist concentration-response curve displacements will approach a limit over a reasonable concentration range and a clear picture of the extent of allosteric modulation might be both obtained and quantified. Furthermore, the common finding that allosteric modulators of muscarinic receptors come from a wide variety of structurally diverse chemicals, often with other pharmacological actions (Tränkle et al 1996), might point to the present approach as a serious alternative for quantifying modulator-antagonist interactions in functional experiments.

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