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Pharmacological estimation of agonist affinity: detection of errors that may be caused by the operation of receptor isomerisation or ternary complex mechanisms

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1 Recent theoretical studies have questioned the pharmacological estimation of agonist affinity. They showed that when receptor isomerisation or ternary complex mechanisms operate, the receptor inactivation method can substantially overestimate affinity, whereas methods for partial agonist analysis are more accurate. We previously suggested that the operation of such mechanisms and therefore the presence of errors could be detected by analysing the same partial agonist by the receptor inactivation and comparative methods. This paper describes the practical application of this test.

2 The ternary complex mechanism was simulated for a partial agonist under various conditions relating receptor (R) and transducer (T) concentrations, one of which also corresponds to the receptor isomerisation mechanism. The theoretical data so generated were then analysed by the inactivation and comparative methods to quantify the magnitude of error of affinity estimation that could occur.

3 This analysis showed that for a partial agonist with approximately 85% of the activity of a full agonist, the inactivation method could produce an affinity (pK_A) estimate up to 0.7 $\log_{10}$ units higher than that produced by the comparative method. This difference would occur when the total receptor concentration ($[R_0]$) is less than or equal to the total transducer concentration ($[T_0]$). It also showed that the overestimation of affinity by the inactivation method was accompanied by drastic overestimation of E_m, the maximal effect parameter.

4 The test was then exemplified using the muscarinic receptor system in the guinea-pig isolated left atrial preparation, where there is evidence that a ternary complex mechanism operates. The test agonist was pilocarpine, which produced on average 83% of the activity of the full agonist, carbachol. Pilocarpine was analysed in comparison with carbachol and by receptor inactivation in the same tissue resulting in small and statistically insignificant differences in E_m (96.7% and 97.3% respectively) and pK_A (5.03 and 4.95 respectively).

5 In conclusion, in this experimental system, there was no evidence for the errors in agonist affinity estimation predicted by theory. Although this conclusion only applies to this system and application of the test to others is necessary to establish the generality of the present results, further examination of the theoretical basis for the predicted errors is required.

Introduction

In a number of recent articles (Colquhoun, 1987; Mackay, 1988; Kenakin, 1989; Leff & Harper, 1989) attention has been drawn to the theoretical unreliability of agonist affinity estimates obtained by pharmacological methods due to the operation of receptor isomerisation (del Castillo & Katz, 1957) and ternary complex (De Lean *et al.*, 1978) mechanisms.

In the case of the receptor isomerisation mechanism it has been shown that it is theoretically impossible, by use of traditional pharmacological methods (Stephenson, 1956; Furchgott, 1966; Barlow *et al.*, 1967), to estimate agonist affinity independently of efficacy, with the consequence that affinity itself is overestimated (Colquhoun, 1987). The same predictions are made in the case of the ternary complex mechanism when the concentration of agonist-receptor complexes is similar to or less than the concentration of transducer units with which they interact (Mackay, 1988; Leff & Harper, 1989). However, it is predicted for both mechanisms that the magnitude of overestimation of affinity will be larger in the case of full agonist analysis by the receptor inactivation method of Furchgott (1966) than in the case of partial agonist analysis by either the interaction method (Stephenson, 1956) or the comparative method (Barlow *et al.*, 1967). It follows, if these theoretical predictions are correct, that when the same agonist (which would necessarily be a partial agonist) is analysed by the inactivation method and either of the two latter methods, different estimates of affinity should be obtained. As suggested previously (Leff & Harper, 1989) this provides an experimental test for the operation of conditions under which erroneous affinity estimates may be made. This test was originally proposed to detect the operation of the ternary complex mechanism but it applies equally well to systems obeying the receptor isomerisation mechanism.

The present paper explains the practicalities of this test and illustrates its potential utility by its application to the analysis of a partial muscarinic agonist.

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Theory

The estimation of agonist affinity by pharmacological methods (Stephenson, 1956; Furchgott, 1966; Barlow *et al.*, 1967) implicitly assumes the validity of traditional receptor theory. With increasing knowledge of receptor mechanisms, it has become necessary to question the utility of traditional receptor theory as a basis for analysing agonist action (Colquhoun, 1987). The central issue is that the traditional theory assumes that affinity can be estimated independently of intrinsic efficacy. Analysis of two apparently plausible mechanisms for the activation of receptors by agonists indicates that this assumption is not valid. Below are given the main predictions of these analyses. For full theoretical details the reader is referred to the original papers.

Receptor isomerisation mechanism

This mechanism was originally proposed to explain how agonists exert their effects in ion-channel-linked receptor

systems (del Castillo & Katz, 1957):

$$A + R \overset{K_A}{\longleftrightarrow} AR \overset{E}{\longrightarrow} AR^*$$

R represents unoccupied receptors, AR represents occupied but unactivated receptors and AR* represents activated receptors. Thus, the generation of a pharmacological effect by the agonist depends on its ability to bind to the receptor and then to elicit its isomerisation into the active state. In this scheme, agonist affinity is determined by the dissociation constant, K_A, for the AR complex, and intrinsic efficacy is defined by the isomerisation constant, E (see Colquhoun, 1987).

Colquhoun (1987) gave the theoretical results of applying pharmacological methods for the estimation of agonist affinity in such systems. In the case of the irreversible receptor inactivation method (Furchgott, 1966), it was predicted that affinity would be overestimated, the estimated dissociation constant being:

$$K_{Aest} = K_A/(1 + E) \quad (1)$$

showing that the extent of overestimation would be proportional to the intrinsic efficacy of the agonist.

In contrast, in the case of the comparative (Barlow *et al.*, 1967) or interaction (Stephenson, 1956) methods for the analysis of partial agonists it was predicted that the estimated affinity would be close to the true value so long as the intrinsic efficacy of the test partial agonist was much less than that of the reference full agonist.

Ternary complex mechanism

This mechanism was originally proposed to explain how agonists exert their effects in receptor systems coupled to G-proteins (De Lean *et al.*, 1978). In principle, it may apply to any system in which the receptor interacts with a transducer unit in order to initiate a response:

$$A + R \overset{K_A}{\longleftrightarrow} AR + T \overset{K_{AR}}{\longleftrightarrow} ART$$

As with the isomerisation mechanism, R represents unoccupied receptors, AR represents occupied but unactivated receptors, and ART represents receptors in their active state due to coupling with transducers. K_A determines affinity in this mechanism as in the case of the isomerisation mechanism, whereas efficacy is now determined by the dissociation constant, K_{AR}, for the ternary complex, ART.

In this case, predictions relating to the application of pharmacological methods for affinity estimation depend on the relative concentrations of R and T (Leff & Harper, 1989). Denoting the total concentrations of these as $[R_0]$ and $[T_0]$ respectively, when $[R_0] \gg [T_0]$, the receptor inactivation method as well as the comparative method provides a correct K_A estimate. When $[R_0] \ll [T_0]$, the comparative method provides a correct estimate but the receptor inactivation method overestimates affinity, the estimated dissociation constant being:

$$K_{Aest} = K_A/(1 + [T_0]/K_{AR}) \quad (2)$$

This result, which was also given by Mackay (1988), is analogous to the prediction made for the isomerisation mechanism (equation 1), the extent of overestimation of affinity being dependent on intrinsic efficacy, in this case $1/K_{AR}$. When $[R_0] = [T_0]$, both the inactivation and comparative methods overestimate affinity but the size of the error is larger for the inactivation method.

A test for errors in affinity estimation

It follows, if these mechanisms operate, that the estimate of affinity for a partial agonist obtained using the receptor inactivation method will, in general, be higher than that obtained by the comparative method. Only in the case of the ternary complex mechanism, with $[R_0] \gg [T_0]$, are the estimates predicted to be the same, as would be assumed by traditional theory. This provides a test which may help to detect the operation of mechanisms which do not accord with traditional receptor theory and in doing so assist in avoiding erroneous affinity estimation. Alternatively, the test may serve to indicate that the traditional theory holds and that the associated affinity estimates are valid.

In the first part of this study, theoretical data are generated corresponding to the two mechanisms, and analysed in order to evaluate the magnitude of difference in affinity estimates that is likely to occur in practice. Having established the detection limits of the test in this way, it is then applied to experimental data obtained in a muscarinic receptor system.

Methods

Computer simulations

Agonist-concentration effect, E/[A], curves were generated with the formulation of the ternary complex model given by Leff & Harper (1989) and the reader is referred to that paper for full theoretical derivations; only brief details will be given here. According to theory, the equilibrium concentration of active ART complexes is given by a quadratic function of agonist concentration:

$$[ART]^2 + b[ART] + c = 0 \quad (3)$$

in which

$$b = -K_{AR}(1 + K_A/[A]) + [T_0] + [R_0]$$

$$c = [R_0][T_0]$$

[ART] has values defined by the roots of this equation. The pharmacologically meaningful one is:

$$[ART] = (-b - \sqrt{b^2 - 4c})/2 \quad (4)$$

E/[A] curves were simulated assuming that E equated with [ART], choosing parameter values to represent the conditions $[R_0] \gg$, = or $\ll [T_0]$.

Under the condition $[R_0] \ll [T_0]$, the [ART]/[A] relation can be written clearly (Leff & Harper, 1989):

$$[ART] = \frac{[R_0]\tau_T[A]}{K_A + (1 + \tau_T)[A]} \quad (5)$$

with τ_T defined as $[T_0]/K_{AR}$.

In the case of the isomerisation mechanism, the relation between [AR*] and [A] can be written (rearranging equation (3) in Colquhoun (1987)):

$$[AR^*] = \frac{[R_0]E[A]}{K_A + (1 + E)[A]} \quad (6)$$

which is clearly analogous to equation (5), the only difference being the mechanistic definition of efficacy.

This means that results obtained simulating equation (4) under the condition $[R_0] \ll [T_0]$ can be taken to represent either the ternary complex or isomerisation mechanisms.

All simulations were carried out on a Compaq 386/25 using the commercially available spreadsheet package, Symphony, produced by Lotus.

Guinea-pig isolated left atria

Male Dunkin-Hartley guinea-pigs (300–400 g) were killed by a blow to the back of the head. The left atria were quickly removed and suspended in 20 ml organ baths containing Krebs solution of the following composition (mM): NaCl 118.41, KCl 4.69, $MgSO_4$ 1.18, KH_2PO_4 1.18, glucose 11.10, $NaHCO_3$ 24.99 and $CaCl_2$ 2.52. This was maintained at 37°C and continually gassed with 5% CO_2 in O_2. The atria were

attached at the base to tissue-hook electrodes by cotton thread and connected at the top to isometric force transducers (Ormed Beam) with steel clips. The diastolic (resting) tension was set at an initial value of 1 g wt. The tissues were electrically stimulated with pulses of 5 ms duration, at a frequency of 2 Hz and a voltage which was 1.5 times that required for a threshold response (twitch). Generated force was recorded on a Devices M19 or an Advance Bryans flat bed recorder.

Experimental protocols

General All tissues were allowed a 60 min period of equilibration before the addition of any drugs. Agonist concentration-effect, E/[A], curves were constructed by cumulative additions of either carbachol or pilocarpine at 0.5 $\log_{10}$ unit increments. Up to 3 E/[A] curves were performed in each tissue. Responses were recorded as percentage inhibitions of the stimulated twitch.

Carbachol efficacy estimation by the receptor inactivation method In each tissue a carbachol E/[A] curve was constructed. After washing, tissues were incubated with the irreversible antagonist phenoxybenzamine (Pbz) (3 μM or 10 μM) or vehicle (40% polyethylene glycol) for 30 min. Following removal of the irreversible antagonist by several changes of the organ bath Krebs solution over a 40 min period, a second carbachol E/[A] curve was performed.

Pilocarpine affinity estimation by the comparative and irreversible receptor inactivation methods In each tissue a carbachol E/[A] curve was constructed, followed, after washing, by a pilocarpine E/[A] curve. Tissues were subsequently washed and then incubated with Pbz (0.3 μM, 0.5 μM or 1 μM) or vehicle for 30 min. Pbz was then removed by several washes over a 40 min period and a further pilocarpine E/[A] curve constructed.

Drugs

The following drugs were used: carbachol chloride (Sigma Chemical Company), pilocarpine hydrochloride (Evans Medical Ltd), phenoxybenzamine hydrochloride (Smith, Kline and French Laboratories), and atropine sulphate (Sigma Chemical Company).

Carbachol, pilocarpine and atropine were dissolved in distilled water. Phenoxybenzamine was dissolved in 40% polyethylene glycol.

Data analysis

Operational model fitting Computer-generated and experimental data were fitted using the operational model of agonism (Black & Leff, 1983; Black *et al.*, 1985):

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (7)$$

in which E and [A] are the pharmacological effect and the concentration of the agonist, respectively; E_m is the maximum possible effect; K_A is the agonist dissociation constant (this was estimated as the negative logarithm, that is, pK_A); τ is the efficacy of the agonist (estimated as a logarithm); n determines the steepness of the occupancy-effect relation.

The fitting procedures employed are described in detail elsewhere (Leff *et al.*, 1989); only brief details will be given here. As a multiple curve design was used, each tissue provided an estimate of K_A by each of the comparative and inactivation methods. In the case of the comparative method the partial agonist curve data were fitted to the operational model (equation 7) simultaneously to fitting the full agonist data to a logistic function of the form:

$$E = \frac{E_m [A]^n}{[A_{50}]^n + [A]^n} \quad (8)$$

where E_m and n are as defined above and $[A_{50}]$ is the location parameter for the full agonist curve. (Location parameters were estimated as negative logarithms ($p[A_{50}]$)). This allows K_A as well as τ for the partial agonist to be estimated as well as E_m and n from each pair of curves.

In the case of the inactivation method, the E/[A] curve data for the agonist (full or partial) before and after receptor inactivation were fitted to equation (7) providing a common estimate of E_m, n and K_A, and a value of τ for each curve in the pair. Average values of the parameter estimates are quoted with standard errors corresponding to between tissue variation. Comparisons of parameter estimates obtained by the comparative and inactivation methods were made by paired *t* test. Standard errors derived from the fitting procedure corresponding to internal fitting error (a measure of goodness-of-fit) are also quoted.

Logistic curve fitting In control experiments designed to determine any effect of drug vehicle on the response to carbachol and pilocarpine, individual E/[A] curves were fitted to a logistic function of the form:

$$E = \frac{\alpha [A]^m}{[A_{50}]^m + [A]^m} \quad (9)$$

where α, $[A_{50}]$ and m are the asymptote, location and slope parameters respectively (any vehicle effect would be shown by significant changes in one or more of these parameters).

All data fitting procedures were carried out with the statistical package, BMDP, and a Vax 11/780 mainframe computer.

Results

Analysis of theoretical data for a partial agonist

Figure 1 shows theoretical E/[A] curves generated using equation (4). Parameter values were chosen to represent the conditions $[R_0] \gg [T_0]$ (panel (a)), $[R_0] = [T_0]$ (panel (b)) and $[R_0] \ll [T_0]$ (panel (c)) in the ternary complex mechanism, the last condition also representing the isomerisation mechanism. Under each condition, an intrinsic efficacy ($1/K_{AR}$) value was chosen to simulate a partial agonist which can generate approximately 85% of the maximum effect. The same agonist was simulated using a lower value of $[R_0]$ representing the effects of receptor inactivation, and a full agonist curve was also generated in order to analyse the partial agonist by the comparative method.

'Data' points covering a realistic experimental range were sampled from the curves at 0.5 $\log_{10}$ unit intervals. These data are shown as symbols in Figure 1. These 'data' points refer to concentrations of [ART]. For curve fitting, [ART] was assumed to be directly proportional to effect, and the concentrations of [ART] were scaled up to cover a range of 0 to 100. E_m for the system is equivalent to the maximum theoretical concentration of [ART] that can be achieved. For each condition, the partial agonist curve was analysed by the comparative method and by the inactivation method. The resulting estimates of affinity and of the other operational parameters are collated in Table 1. This analysis showed that, under the condition $[R_0] \gg [T_0]$, accurate estimates of pK_A were obtained by both methods. Also, it is to be noted that E_m was accurately estimated by both methods and the standard errors associated with the estimated model parameters were small and similar. However, under the conditions $[R_0] = [T_0]$ and $[R_0] \ll [T_0]$, the inactivation method overestimated pK_A by some 0.7 $\log_{10}$ units, whilst the comparative method was

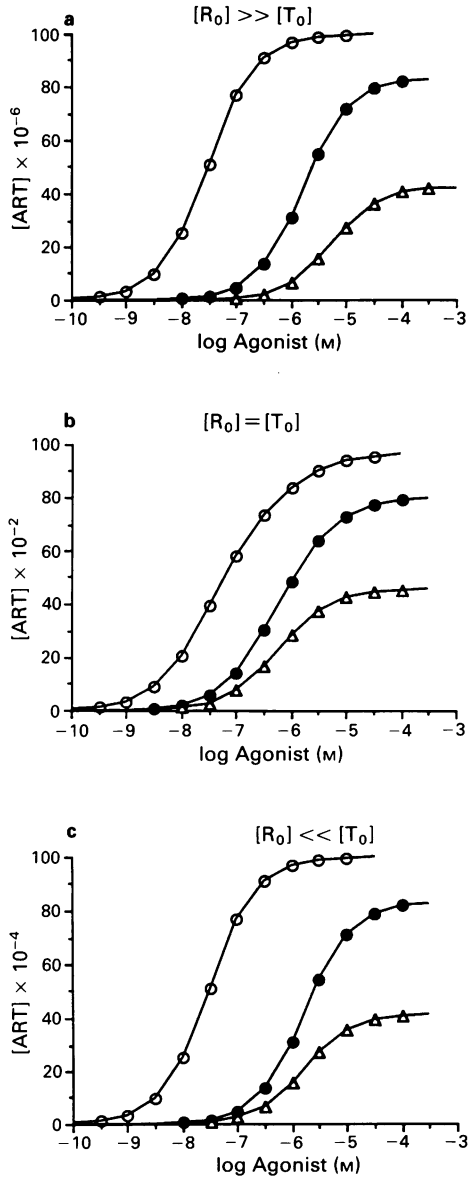

Figure 1 Theoretical E/[A] curves under various conditions relating receptor ($[R_0]$) and transducer ($[T_0]$) concentrations. Panels (a), (b) and (c) represent the conditions $[R_0] \gg [T_0]$, $[R_0] = [T_0]$ and $[R_0] \ll [T_0]$ respectively. The latter condition is equivalent to the isomerisation mechanism. Under each condition, theoretical curves were generated corresponding to a full agonist (○), a partial agonist (●) with approximately 85% of the activity of the full agonist and the same partial agonist after receptor inactivation (△).

The simulation parameters used were as follows:

	Affinity		*Intrinsic efficacy*				
	pK_A full	pK_A partial	pK_{AR} full	pK_{AR} partial	$[T_0]$	$[R_0]$ initial	$[R_0]$ inactivated
(a)	4.5	5.0	3.0	0.7	0.0001	1.0	0.15
(b)	4.5	5.0	3.0	1.3	1.0	1.0	0.5
(c)	4.5	5.0	3.0	0.7	1.0	0.01	0.005

The symbols represent the 'data' points that were sampled from computer generated curves and analysed by operational model-fitting (see Table 1).

accurate to within 0.1 $\log_{10}$ units. Also, E_m was overestimated by up to 500% by the inactivation method but accurately estimated to within 5% by the comparative method under both conditions. Furthermore, the standard errors associated with model fitting were considerably larger for the inactivation method than for the comparative method (see Table 1). For example under the condition $[R_0] = [T_0]$ the standard error on E_m was about 50%, whereas under the condition $[R_0] \gg [T_0]$ it was less than 1%. This is an indication of a poor quality of fit, which is anticipated since the theoretical data should not accord with the operational model under the conditions $[R_0] \leqslant [T_0]$. Indeed, when $[R_0] \ll [T_0]$ standard errors could not be estimated due to poorness of fit.

Table 1 Analysis of theoretical data for a partial agonist by comparative and inactivation methods

	Comparative Method		*Inactivation Method*	
	Estimate	*Standard error*	*Estimate*	*Standard error*
$[R_0] \gg [T_0]$				
E_m	98.9	0.4	98.6	0.8
n	1.02	0.01	1.03	0.02
log τ	0.69	0.01	0.70	0.02
pK_A	5.02	0.02	5.01	0.02
$[R_0] = [T_0]$				
E_m	95.0	0.8	342.6	175.7
n	0.75	0.02	0.56	0.08
log τ	0.96	0.04	−0.94	0.97
pK_A	5.10	0.05	5.72	0.26
$[R_0] \ll [T_0]$ (also represents the isomerisation mechanism)				
E_m	100.1	0.2	555.7	—
n	0.99	0.01	0.98	—
log τ	0.71	0.01	−0.77	—
pK_A	4.98	0.02	5.69	—

In the last fit, standard errors could not be computed due to degeneracies encountered during the fitting procedure. The 'correct' estimates of pK_A and E_m were 5.00 and 100.0 respectively.

Analysis of experimental data for the full agonist, carbachol

In order to apply the comparative method to the analysis of the partial agonist, pilocarpine (see below), it was necessary first to ensure that the reference agonist, carbachol, acts as a full agonist in the guinea-pig left atrial preparation. E/[A] curves for carbachol were obtained before and after Pbz-treatment (3 μM or 10 μM, 30 min). Control experiments showed that consecutive carbachol E/[A] curves obtained in the absence of Pbz treatment were effectively superimposed, confirming the validity of the paired curve analysis. The mean logistic parameters (± s.e.; $n = 5$) of these curves were as follows: 1st curve: $\alpha = 86.9$ (± 1.7); $m = 1.35$ (± 0.07); $p[A_{50}] = 6.68$ (± 0.04). 2nd curve: $\alpha = 85.8$ (± 1.5); $m = 1.40$ (± 0.06); $p[A_{50}] = 6.62$ (± 0.04).

Figure 2 illustrates typical results obtained in a single Pbz-treated tissue. The lines drawn through the data are the results of operational model-fitting. The substantial rightward shift and depression of the carbachol E/[A] curve produced by Pbz treatment indicated that the agonist has a high efficacy in this tissue. Analysis of 7 experiments gave an average estimate of efficacy (τ) of 72.4 ($\log \tau = 1.86$ (± 0.11)) and an estimate of affinity (pK_A) of 4.73 (± 0.10). The high efficacy confirmed that carbachol was indeed a full agonist in this system. This is in agreement with the findings of other workers (Furchgott, 1966). The reliability of this analysis and that with pilocarpine requires that Pbz acts purely to decrease $[R_0]$ in this system. This was confirmed by experiments in which coincubation with the reversible competitive antagonist atropine was shown to protect the tissues from the effects of Pbz (data not shown, $n = 3$). Thus, E/[A] curves for carbachol obtained following incubation of tissues with atropine (0.1 μM) and Pbz (10 μM) then washout were indistinguishable from curves obtained following atropine treatment alone.

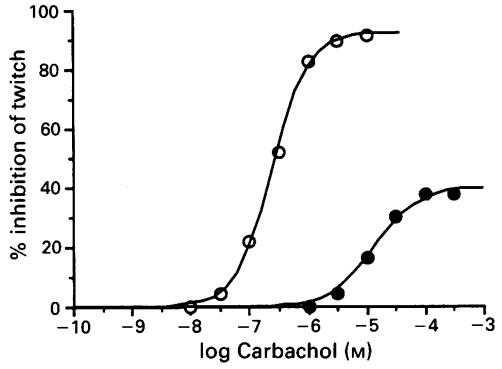

Figure 2 Effect of phenoxybenzamine (Pbz) treatment on carbachol E/[A] curves. Carbachol E/[A] curves were obtained before (○) and following 30 min exposure to 10 μM (●) Pbz. The symbols represent the data from a single tissue. The lines drawn through the data are the results of operational model fitting using the receptor inactivation method. For this particular tissue the estimated model parameters were as follows (standard errors in parentheses): E_m = 92.6 (± 1.3); n = 1.43 (± 0.08); τ_1 (control) = 58.9 (log τ_1 = 1.77 (± 0.07)); τ_2 (10 μM Pbz) = 0.8 (log τ_2 = −0.08 (± 0.04)); pK_A = 4.84 (± 0.07).

Analysis of experimental data for the partial agonist, pilocarpine

Pilocarpine E/[A] curves were obtained before and after Pbz treatment, following construction of an E/[A] curve for carbachol. As above, control experiments showed no differences between consecutive pilocarpine E/[A] curves obtained in the absence of Pbz treatment. The mean logistic parameters (± s.e.; n = 6) were as follows: 1st curve: α = 67.9 (± 4.8); m = 1.21 (± 0.10); $p[A_{50}]$ = 5.47 (± 0.07). 2nd curve: α = 68.6 (± 2.9); m = 1.40 (± 0.13); $p[A_{50}]$ = 5.42 (± 0.05).

Figure 3 illustrates a typical set of data obtained in a single Pbz-treated left atrium. Eight such experiments were conducted. In each instance, pilocarpine was analysed in comparison with carbachol and by the inactivation method.

The lines drawn through the data are the results of operational model-fitting. Table 2 shows the resulting parameter estimates and statistical analysis of the pK_A and E_m values obtained. Evidently, for each of these parameters the difference between the estimates provided by the two methods was very small and clearly not significant. Also, fitting errors associated with the pK_A and E_m estimates were small, on average 3% and 8% for the inactivation method and 2% and 2% for the comparative method (individual errors not shown).

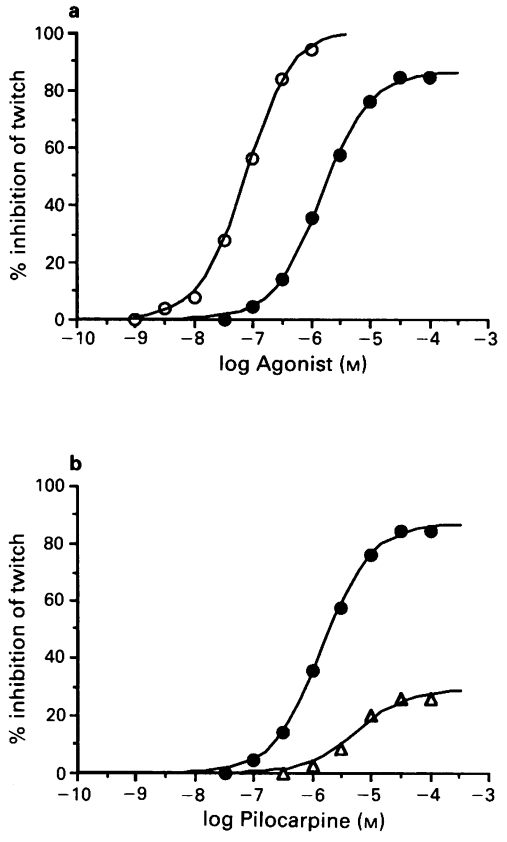

Figure 3 Analysis of pilocarpine E/[A] curve data by the comparative and inactivation methods. The data shown are from a single tissue for carbachol (○), pilocarpine (●) and pilocarpine after 30 min exposure to 1 μM phenoxybenzamine (Pbz, △). The lines drawn through the data are the results of operational model-fitting. Panel (a) illustrates the comparative analysis of pilocarpine using carbachol as the reference full agonist. The model parameter estimates were as follows (standard errors in parentheses): E_m = 101.0 (± 2.1); n = 1.08 (± 0.05); τ = 5.1 (log τ = 0.71 (± 0.05)); pK_A = 5.06 (± 0.06). Panel (b) illustrates the analysis of pilocarpine by receptor inactivation. The model parameter estimates were as follows (standard errors in parentheses): E_m = 105.6 (± 7.0); n = 1.06 (± 0.08); τ_1 (control) = 4.3 (log τ_1 = 0.63 (± 0.14)); τ_2 (1 μM Pbz) = 0.4 (log τ_2 = −0.40 (± 0.07)); pK_A = 5.12 (± 0.13).
For complete results see Table 2.

Discussion

The possibility that errors may occur in the estimation of agonist affinity by pharmacological methods makes it important that tests are available for their detection. This paper illustrates one such test.

Theoretical studies (Colquhoun, 1987; Mackay, 1988; Leff & Harper, 1989) of two plausible receptor models, the isomerisation (del Castillo & Katz, 1957) and the ternary complex (de Lean *et al.*, 1978) mechanisms, had already shown that the receptor inactivation method (Furchgott, 1966) is likely to provide higher estimates of agonist affinity than the interaction (Stephenson, 1956) or comparative (Barlow *et al.*, 1967) methods. It had also been suggested (Leff & Harper, 1989) that this difference could serve as the basis for a test to detect the operation of such receptor mechanisms and, therefore, to assess the validity of agonist affinity estimates obtained by pharmacological methods.

In practice, such a test can only be applied to partial agonists since only these can be analysed by both the inactivation and comparative methods. The first objective of the present study was to establish the magnitude of the difference in affinity estimates that the two tests would be likely to provide. This was done using theoretical data for a partial agonist operating by the ternary complex and isomerisation mechanisms and producing approximately 85% of the maximum possible effect. This effect level was chosen to be similar to that produced by the experimental example, pilocarpine. This

Table 2 Mean parameter estimates and statistical analysis of E_m values and pilocarpine pK_A values obtained from experimental data

	Comparative method		*Inactivation methods*		*Mean difference*	
	E_m	pK_A	E_m	pK_A	E_m	pK_A
Mean (n = 8)	96.7	5.03	97.3	4.95	0.6	0.08
s.e.	3.3	0.08	5.4	0.10	3.6	0.15
paired t test					$P > 0.5$	$P > 0.5$

theoretical analysis showed that, other than under the condition $[R_0] \gg [T_0]$ in the ternary complex model, the inactivation method was likely to produce an affinity estimate some five fold or 0.7 $\log_{10}$ units higher than the true value, whereas the comparative method was accurate to within 0.1 $\log_{10}$ units under all conditions. In addition, the overestimation of affinity by the inactivation method was accompanied by drastic overestimation of E_m. Therefore, in the context of the two mechanisms studied, it can be concluded that the operation of conditions unfavourable to affinity estimation can be detected by differences both in the values of K_A and E_m obtained by the two pharmacological methods.

In model-fitting terms, the overestimation of K_A and E_m are connected. The result of overestimating affinity is that the location of the control curve, obtained in the absence of receptor inactivation, is nearer to the estimated K_A than to the true K_A. Therefore, the partial agonist is treated by the fitting process as if it has lower efficacy than it actually does. In other words, the maximum effect of the partial agonist is treated as if it is lower in relation to the maximum possible effect, E_m, than it really is. Therefore, conversely, E_m is overestimated. This also accounts for the rather large standard error associated with E_m under the conditions $[R_0] = [T_0]$ and the inability to fit under the condition $[R_0] \ll [T_0]$. The lower the maximum effect of the partial agonist, the more the fitting process will have difficulty in projecting E_m from the curve depression caused by receptor inactivation.

The second objective of this study was to exemplify the test in a practical example. The muscarinic receptor system in the guinea-pig left atrium was chosen since there is evidence that it is a G-protein-linked system (Eglen *et al.*, 1987) and, therefore, could be anticipated to operate by the ternary complex mechanism. In this system, as demonstrated here, carbachol is a full agonist (see Figure 2 and analysis), permitting its use as the reference agonist in the comparative method. Pilocarpine demonstrates partial agonism in this tissue, achieving approximately 85% of the maximum effect of carbachol. Analysis of pilocarpine by receptor inactivation and by comparison with carbachol produced very similar, and statistically indistinguishable, estimates of pK_A (4.95 and 5.03 respectively) and E_m (97.3 and 96.7 respectively).

Also the standard errors associated with the parameters, in particular E_m, were small, giving no indication of the problems of goodness-of-fit associated with analysis of theoretical data.

Therefore, in this practical example there was no indication of the errors which the operation of the ternary complex mechanism can, in theory, produce. Waud (1969) performed a similar study on pilocarpine using the guinea-pig ileum preparation. He obtained a pK_A of 5.03 by the inactivation method and one of 5.36 by the comparative method. Although that study was not conducted with the above issues in mind, it confirms our findings.

The absence of the predicted theoretical errors in the present study does not rule out that the mechanism applies since, under one condition ($[R_0] \gg [T_0]$), similar estimates of pK_A and E_m are predicted by theory. If this is the correct explanation, the results would imply that although Pbz must have altered the ratio between $[R_0]$ and $[T_0]$ the reduction could not have been sufficient for $[R_0]$ to then approximate to $[T_0]$. That is, the condition $[R_0] \gg [T_0]$ would have applied before and after receptor inactivation. Of course, this is not to say that the same initial ratio between $[R_0]$ and $[T_0]$ would apply in other receptor systems and in such systems where $[R_0] \leqslant [T_0]$ the theoretically predicted errors may be demonstrable.

The results of the present study prompt the question as to whether the theoretical objections raised about agonist affinity estimation by pharmacological methods are justified since one possible interpretation of these results is that the theories and, therefore, their predictions are incomplete or wrong, as discussed elsewhere (Leff *et al.*, 1990). Clearly, further application of experimental tests to detect errors in agonist quantification and increased understanding of receptor-effector mechanisms will do much to clarify the issue.

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