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Estimation of competitive antagonist affinity from functional inhibition curves using the Gaddum, Schild and Cheng-Prusoff equations

[1]S. Lazareno & *N.J.M. Birdsall

MRC Collaborative Centre, 1–3 Burtonhole Lane, Mill Hill, London NW7 1AD and *Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA

1 The estimation of antagonist affinity from functional experiments in which the effect of a fixed agonist concentration is reduced by a range of antagonist concentrations ('functional inhibition curves') has been considered from both a theoretical and experimental viewpoint.

2 Theoretical predictions are compared with results obtained from the stimulation of [^{35}S]-GTPγS binding by acetylcholine to membranes of Chinese hamster ovary (CHO) cells stably transfected with human m1–m4 muscarinic receptors, and inhibition of the stimulated binding by pirenzepine and AQ-RA 741.

3 The usual procedure of applying the Cheng-Prusoff correction is shown to be theoretically invalid, and predictions are made of the size and distribution of errors associated with this procedure.

4 A different procedure for estimating antagonist affinity, using the principles of dose-ratio analysis and analogous to use of the Gaddum equation, is found to be accurate and theoretically valid.

5 A novel method of analysis allows accurate estimation of both antagonist affinity and Schild slope, by fitting the combined data from an antagonist inhibition curve and an agonist activation curve directly to a form of the Schild equation (derived by Waud) using non-linear regression analysis.

6 It is shown that the conventional Schild analysis can be enhanced by treating part of the data as a family of inhibition curves and including in the Schild plot dose-ratios estimated from the inhibition curves.

Keywords: Muscarinic receptor subtypes; GTPγS binding; antagonist affinity, dose-ratio analysis; receptor theory

Introduction

Antagonist affinity is frequently estimated in binding studies by measuring the inhibition of binding of a fixed concentration of radioligand and converting the observed IC_{50} (concentration of antagonist inhibiting binding by 50%) to a K_i (estimate of antagonist dissociation constant) with the equation of Cheng & Prusoff (1973). This equation assumes that both agents interact in a reversible competitive fashion at a single site according to the law of mass action, that the reaction is at equilibrium, and that the free concentrations of the agents are known and constant throughout the experiment. The K_d of the radioligand must also be known.

In functional studies the dissociation constant of the agent providing the signal, the agonist, is not known, and the relationship between observed response and agonist occupancy is also not known. In order to overcome these limitations, null methods have been devised which involve the same assumptions as in the binding experiments and, in addition, the assumption that a particular response reflects a certain level of agonist occupancy regardless of the level of antagonist occupancy. The central feature of these methods is the measurement of 'dose ratios', ratios of agonist concentrations in the presence and absence of antagonist which produce the same response. The simplest such method uses the Gaddum equation (Gaddum, 1957) and involves the measurement of agonist effect over a range of concentrations in the absence and presence of a fixed concentration of antagonist. The method of Arunlakshana & Schild (Schild, 1957) uses dose-ratios obtained in the presence of a number of antagonist concentrations.

The experimental designs which are used in Gaddum and Schild analyses typically involve the construction of two or more agonist concentration-effect curves in the absence and presence of fixed antagonist concentrations, for the practical reason that most whole tissues produce a rapid response to agonist but do not maintain a stable response to a fixed agonist concentration for the often long periods of time needed for equilibration of antagonist. Some functional responses, however, notably those measured biochemically, readily lend themselves to designs involving an antagonist titration in the presence of a fixed concentration of agonist, an 'inhibition curve' design.

Although the application of inhibition curve design to Gaddum analysis (Lazareno & Roberts, 1987) and, recently, to Schild analysis (Pöch *et al.*, 1992) have been described, most authors have been content to analyse functional inhibition curves with the Cheng-Prusoff equation (e.g. Ford *et al.*, 1992), despite the fact that the parameters and assumptions of this analysis are not applicable to functional experiments. The Cheng-Prusoff equation, however, is virtually identical to the null-method equation of Lazareno & Roberts (1987) at high agonist concentrations, so under this condition the Cheng-Prusoff equation should yield the correct result. Eglen & Whiting (1989) have explicitly investigated the applicability of the Cheng-Prusoff equation to functional data and have reached the opposite conclusion: they claimed to demonstrate, both experimentally and theoretically, that the equation may, by chance, yield the correct result with low fixed agonist concentrations, but results in serious errors when used with high agonist concentrations. If this result were correct, it would invalidate the null-method inhibition-curve type of design of Lazareno & Roberts (1987) and Pöch *et al.* (1992).

Here we demonstrate (i) the validity of Gaddum analysis as applied to inhibition curves, (ii) the direct fitting of the Schild equation (Waud, 1975; 1976) to single inhibition curves to yield estimates of both Schild slope and pK_b, and (iii) the possibility of enhancing the power of the normal

[1] Author for correspondence.

Schild analysis by extracting dose-ratio values from inhibition curves as well as activation curves. We also explore the validity of the Cheng-Prusoff equation in functional experiments using a theoretical model of agonism, the Operational Model of Black & Leff (1983). The functional measure we have used is the agonist stimulated binding of [^{35}S]-GTPγS to membranes from Chinese hamster ovary (CHO) cells stably transfected with the m1–m4 human muscarinic receptors.

Some of these data have been presented in preliminary form (Lazareno *et al.*, 1993).

Methods

Cell culture and membrane preparation

CHO cells stably expressing human m1–m4 muscarinic receptors (Buckley *et al.*, 1989) were generously provided by Dr N. Buckley (N.I.M.R., London). These were grown in MEM-alpha medium (GIBCO) containing 10% (v/v) new born calf serum and harvested by scraping the cells in a hypotonic medium (20 mM HEPES + 10 mM EDTA, pH 7.4). Membranes were prepared at 0°C by homogenization with a Polytron followed by centrifugation (40,000 *g*, 15 min), were washed once in 20 mM HEPES + 0.1 mM EDTA, pH 7.4, and were stored at − 70°C in the same buffer. The yields of receptor were 3, 1, 3 and 4 pmol mg^{-1} of total membrane protein at m1, m2 m3 and m4 subtypes respectively.

[^{35}S]-GTPγS binding

Membranes were suspended in a buffer containing 20 mM HEPES, 100 mM NaCl and 10 mM $MgCl_2$, pH 7.4 at a protein concentration of 25–50 μg ml^{-1}. Polystyrene tubes (5 ml) containing 1 ml of membrane suspension were incubated with GDP, ACh and antagonist at 30°C for 20 min and then transferred to ice for 15 min. [^{35}S]-GTPγS was added to a final concentration of 100 pM and the samples incubated for 30 min at 30°C. The samples were filtered over glass fibre filters (Whatman GF/B) using a Brandel cell harvester and washed with 5 ml water. The filter discs were extracted overnight in 3 ml scintillant and counted by liquid scintillation spectrometry at an efficiency of about 97%. Assays were conducted in duplicate, with each set of replicates filtered together.

Reagents

[^{35}S]-GTPγS was purchased from Du Pont; acetylcholine (ACh), pirenzepine (PRZ) and GDP were from Sigma, and AQ-RA 741 (11-({4-[4-(diethylamino)-butyl]-1-piperidinyl} acetyl))-5,11-dihydro-6H-pyrido(2,3-b)(1,4)-benzodiazepine-6-one) was a gift from Dr Karl Thomae GmbH.

Data analysis

In Experiment 1 the d.p.m. were not transformed. In Experiment 2 there were small but consistent differences between the 1st and 2nd replicates of the m3 and m4 assays, caused perhaps by small differences in the incubation time or washing procedure, so for all subtypes each set of replicates was expressed as a % of basal activity: this transformation resulted in a small improvement in the coefficient of variation.

The data were analysed by non-linear regression analysis with two programmes. The direct fits to the Schild equation and simulations were conducted with SigmaPlot (Jandel Scientific, Germany), which was also used to produce all the graphs. The logistic fitting utilised Allfit (De Lean *et al.*, 1978, a gift from Dr Munson, NIH); SigmaPlot could have been used, but Allfit was more convenient as it is designed specifically for the analysis of families of logistic curves, with the option of sharing or fixing some or all of the fitted parameters between curves, and testing the statistical validity of such constraints. In all cases the statistical validity of sharing parmeters, where appropriate, was assessed by the 'extra sum of squares' test (Munson & Rodbard, 1980) and a significance level of $P<0.05$.

Cheng-Prusoff analysis

Agonist activation curves and antagonist inhibition curves in the presence of a fixed agonist concentration [A] were fitted to logistic functions to yield the parameters EC_{50} and IC_{50}. The Cheng-Prusoff estimate of dissociation constant (K_{CP}) was obtained with the formula.

$$K_{CP} = IC_{50}/([A]/EC_{50} + 1).$$

A theoretical consideration of the validity of this analysis in functional experiments is contained in the Appendix.

Analysis of inhibition curves using Gaddum analysis

The procedure has been described by Lazareno & Roberts (1987). Gaddum analysis involves the estimation of equiactive agonist concentrations of agonist in the presence and absence of a certain antagonist concentration, and insertion of the ratio of the former to the latter agonist concentration (the 'dose-ratio') in the equation

$$K_b = [B]/(\text{dose-ratio} - 1),$$

where K_b is the estimate of antagonist K_d and [B] is the concentration of antagonist.

Both the traditional and inhibition curve designs contain a titration of agonist alone, to which a second titration is related. In the traditional design the antagonist concentration is fixed by the experimenter, a second agonist titration is performed in its presence, and analysis of the two curves allows an estimation of the equieffective agonist concentration in the presence of antagonist (measured typically at the 50% response level). In the inhibition curve design the equieffective agonist concentration is fixed by the experimenter, the antagonist concentration reducing its effect by 50% is estimated from an antagonist titration in the presence of the fixed agonist concentration, and the agonist concentration causing the same effect (50% that of the fixed concentration) is estimated from the agonist titration. As in the conventional design, there are two titrations which provide estimates for the analysis.

The antagonist inhibition curve and the agonist activation curve containing agonist concentrations *up to the fixed concentration used in the inhibition curve*, were fitted simultaneously to a logistic model with the maxima and minima either defined (Experiment 1) or shared (Experiment 2) (the pK_b estimates were almost identical with either type of constraint) to yield an IC_{50}* for the antagonist and an EC_{50}* for the agonist. The IC_{50}* is the concentration of antagonist which produces, in the presence of the fixed agonist concentration, a response level of 50% of the response obtained with the fixed agonist concentration alone. The EC_{50}* is the agonist concentration which, by itself, produces a response level of 50% of the response obtained with the fixed agonist concentration alone. Note that the EC_{50}* will not be the same as the true EC_{50} unless the fixed agonist concentration itself produces the E_{max} response, and the IC_{50}* is not necessarily identical to the true IC_{50} estimated without constraints. The necessary condition of the analysis is that the antagonist IC_{50}* in the presence of the fixed agonist concentration and the agonist EC_{50}* correspond to the same response level. (The analysis was done here with logistic curve fitting, but Hill analysis, using a logit-log transformation and defined maxima and minima, would have done just as well). The Gaddum equation can now be expressed as

$$K_b = IC_{50}^*/([A]/EC_{50}^* - 1),$$

where [A] is the fixed agonist concentration. Note that at

high agonist concentrations, ($EC_{50}^* \approx EC_{50} << [A]$) this equation is almost identical to the Cheng-Prusoff equation.

Schild analysis

Schild plots were constructed by plotting log (dose-ratio − 1) agonist log([B]), where 'dose-ratio' is the ratio of equiactive agonist concentrations in the presence and absence of antagonist concentration [B]. The plots were analysed by linear regression and, when it was established that the plots were linear with slopes not significantly different from unity, the intercept from the regression analysis with slope constrained to unity was taken as the pK_b estimate.

Direct fitting of data to the Schild model

It is worthwhile reviewing this powerful and underused equation which was first derived by Waud (1975, 1976). If an agonist causes a 50% effect at a concentration of $[EC_{50}^0]$ in the absence of antagonist, and at a concentration of $[EC_{50}]$ in the presence of a fixed concentration [B] of antagonist, then the dose ratio (dr) is $[EC_{50}]/[EC_{50}^0]$, and

$$[EC_{50}] = dr.[EC_{50}^0]. \quad (1)$$

The Schild model relates the dose-ratio to [B] according to the equation $\log(dr-1) = s.\log[B] - \log(K_b)$, where K_b is the antagonist dissociation constant and s is the Schild slope, so

$$dr = ([B]^s/K_b) + 1. \quad (2)$$

If the agonist curves can be described with a logistic function, then effect = (E_{max}-basal) / $(1 + ([EC_{50}]/[A])^b)$ + basal, where b is the slope factor of the agonist curve and [A] is the agonist concentration. Substituting (1) and (2) we obtain the Waud equation

$$\text{Effect} = (E_{max}\text{-basal})/\left(1 + \left\{\frac{[EC_{50}^0] \cdot \left(\frac{[B]^s}{K_B} + 1\right)}{[A]}\right\}^b\right) + \text{basal} \quad (3)$$

A slightly different equation has been used by Waud *et al.* (1978)

$$\text{Effect} = (E_{max}\text{-basal})/\left(1 + \left\{\frac{[EC_{50}^0] \cdot \left(\left(\frac{[B]}{K_B}\right)^s + 1\right)}{[A]}\right\}^b\right) + \text{basal} \quad (4)$$

Here the slope s represents a logistic slope factor of the antagonist occupancy function rather than the molecularity of the antagonist-receptor interaction. Both equations yield identical parameter estimates when fitted to experimental data, except for the estimate of K_b. Small variations in s can lead to quite large variations in K_b when equation (3) is used, but minor variations when (4) is used, and K_b is estimated with greater precision (and less correlation with s) by (4). The Schild slope can only be assigned molecular significance if it is an integer, otherwise it indicates some deviation from the underlying assumptions of the model, and the K_b estimate can only be considered as an estimate of antagonist potency. If the Schild slope is not significantly different from 1 then the best estimate of K_b is obtained by constraining the Schild slope to 1 (as with all the data reported here), but if this is not valid then equation (4) will yield a better estimate of antagonist potency.

Results

Theoretical

Errors caused by the use of the Cheng-Prusoff equation Figure 1 shows the Cheng-Prusoff estimate of dissociation constant divided by the true dissociation constant (K_{CP}/K_i) as a function of log(fixed agonist concentraton/EC_{50}) for various

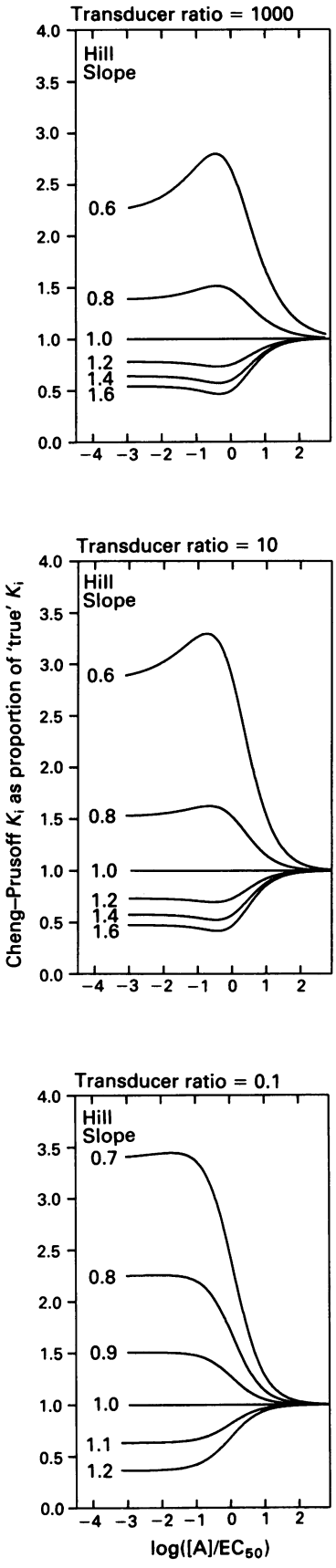

Figure 1 Theoretical error in the use of the Cheng-Prusoff equation to estimate antagonist affinity from IC_{50} values obtained from functional inhibition experiments. 'Cheng-Prusoff' K_i estimates were derived from an 'operational model' of agonist effect as described in the Appendix. Errors (K_{CP}/K_i) are shown as a function of log(fixed agonist concentration/EC_{50}), the Hill slope factor of the logistic function relating [agonist] to effect (see Appendix), and the transducer ratio, τ, which corresponds roughly to receptor reserve.

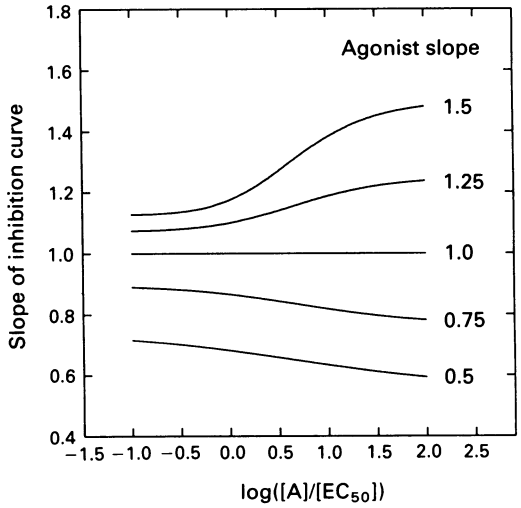

Figure 2 Logistic slope factors of theoretical antagonist functional inhibition curves derived from simulations with the Waud equation using various agonist slope factors. The antagonist Schild slopes were fixed at 1.

values of Hill (logistic) slope of the agonist curve and τ (transducer ratio, related to 'receptor reserve'). When the Hill slope is 1, or when the fixed agonist concentration is 100 fold or more greater than the EC_{50}, the Cheng-Prusoff equation provides a good estimate of the 'true' antagonist K_d. Errors arise when lower agonist concentrations are used and the Hill slope deviates from 1. The magnitude of the error increases as the Hill slope deviates from 1, and for a particular Hill slope, increases with decreasing receptor reserve. The limiting cases, with very large or very small [A], are as described in the Appendix. In addition, a range of fixed agonist concentrations give larger deviations than when [A] is very small, the largest deviation occurring with ($EC_{50}/10 < [A] < EC_{50}$).

Slopes of antagonist inhibition curves If the agonist curve is truly a logistic function with slope $\neq 1$, then the antagonist inhibition curve cannot be a logistic function, though inaccuracies in fitting such inhibition curves to a logistic function are likely to be trivial. Simulated inhibition curves with Schild slopes of 1 were generated using the Waud equation at various levels of $[A]/[EC_{50}]$ and agonist slope. The results in Figure 2 demonstrate that inhibition curves only have slope factors of 1 if the agonist curve is itself rectangular hyper-

Table 1 Parameters for acetylcholine (ACh) and pirenzepine (PRZ) from analysis of Experiment 1

	m1	*m2*	*m3*	*m4*
Fixed [PRZ] concentration (M)	3×10^{-8}	3×10^{-7}	3×10^{-7}	10^{-7}
	10^{-7}	3×10^{-6}	10^{-6}	10^{-6}
	3×10^{-7}	3×10^{-5}	3×10^{-6}	10^{-5}
Analysis				
1 ACh alone pEC_{50} (− log M)	6.10	7.39	5.59	6.91
4 ACh alone pEC_{50} (− log M)	6.10	7.41	5.57	6.91
1 ACh logistic slope	0.53	0.73	0.97	0.75
4 ACh logistic slope	0.55	0.72	0.97	0.75
1 Schild slope	1.14	1.03	0.84	1.03
2 Schild slope	1.12	0.96	0.83	0.90
3 Schild slope	1.13	1.01	0.84	0.97
4 Schild slope	1.15	1.00	0.84	1.03
1 pK_b	8.37	6.60	6.77	7.65
2 pK_b	8.37	6.64	6.85	7.73
3 pK_b	8.38	6.62	6.81	7.69
4 pK_b	8.39	6.66	6.74	7.66

Analysis: (1) activation curves fitted to logistic function, Schild plots from dose-ratios; (2) inhibition curves and corresponding activation curve fitted to logistic with fixed limit parameters, Schild plots from dose-ratios; (3) dose-ratios from (1) and (2) combined in Schild plots; (4) direct fit of dataset to Waud equation; Schild slope estimated from unconstrained analysis, pK_b (and control ACh slope and pEC_{50}) estimated from analysis with Schild slope set to 1.

Table 2 Parameters for acetylcholine (ACh), pirenzepine (PRZ) and AQ-RA 741 from analysis of Experiment 2

	m1	*m2*	*m3*	*m4*
Fixed [ACh] (M)	3×10^{-5}	3×10^{-6}	10^{-4}	3×10^{-6}
Analysis				
1 ACh slope	0.50	0.64	0.84	0.68
3 ACh slope	0.47	0.65	0.85	0.70
1 ACh pEC_{50} (− log(M)	5.69	7.31	5.43	6.97
3 ACh pEC_{50} (− log(M)	5.66	7.31	5.43	6.97
1 PRZ logistic slope	0.71	0.72	0.84	0.74
3 PRZ Schild slope	1.08	0.96	0.95	1.00
1 PRZ pIC_{50}	6.78	4.70	5.30	6.11
2 PRZ pK_b	8.46	6.66	6.81	7.76
3 PRZ pK_b	8.37	6.65	6.79	7.74
4 PRZ pK_{CP}	7.97	6.50	6.75	7.58
1 AQ-RA logistic slope	0.56	0.78	0.86	0.77
3 AQ-RA Schild slope	0.93	1.10	1.03	1.07
1 AQ-RA pIC_{50}	6.56	7.12	5.86	7.01
2 AQ-RA pK_b	8.28	9.09	7.38	8.65
3 AQ-RA pK_b	8.21	9.09	7.34	8.63
4 AQ-RA pK_{CP}	7.76	8.92	7.31	8.47

Analysis: (1) full activation and inhibition curves fitted to logistic function; (2) inhibition curve and corresponding activation curve up to the fixed concentration fitted to logistic with shared limit parameters, IC_{50}* and EC_{50}* values applied to the Gaddum equation; (3) direct fit of inhibition curve and corresponding activation curve to the Waud equation; Schild slope estimated from unconstrained analysis, pK_b (and control ACh slope and pEC_{50}) estimated from analysis with Schild slope set to 1; (4) parameters from analysis (1) applied to Cheng-Prusoff equation.

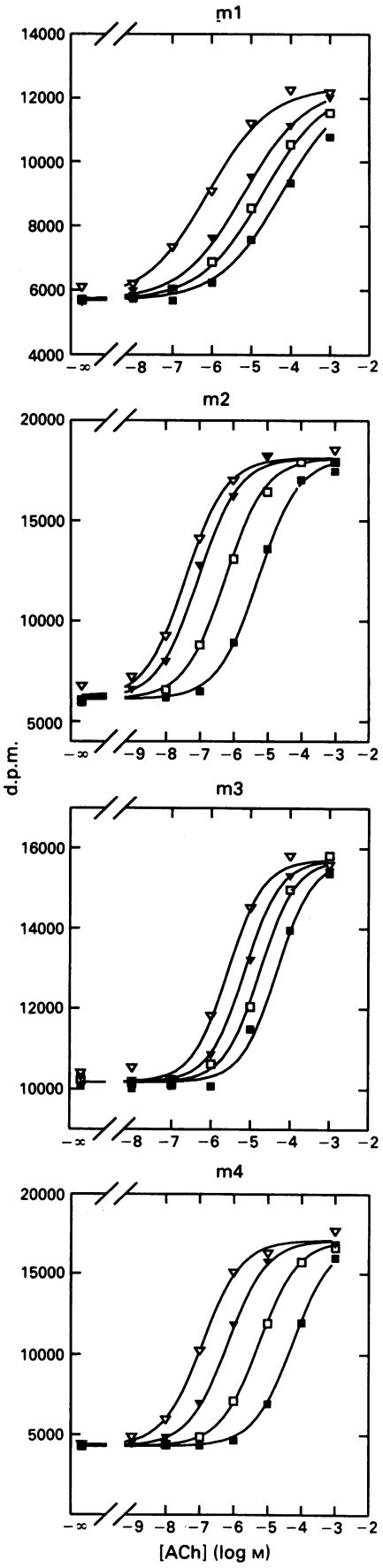

Figure 3 Experiment 1: effect of acetylcholine (ACh) in the presence of various concentrations of pirenzepine on [^{35}S]-GTPγS binding to membranes from CHO cells transfected with m1–m4 muscarinic receptors. The lines show the fit to the Waud equation with Schild slope fixed at 1. Each point is the mean of duplicate observations. The following pirenzepine concentrations were used (M):

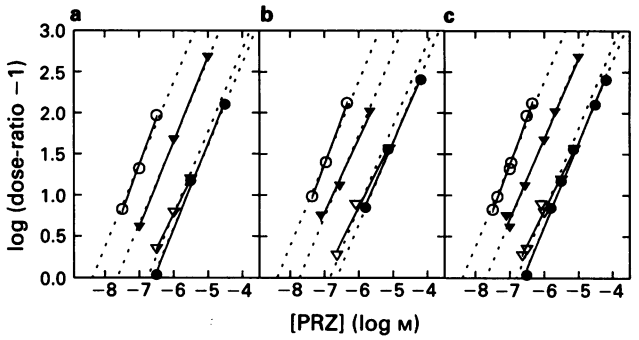

Figure 4 Schild plots of the effect of pirenzepine (PRZ) on acetylcholine potency in stimulating GTPγS binding via m1 (○), m2 (●), m3 (▽) and m4 (▼) receptors (Experiment 1). (a) Dose-ratios derived from EC_{50} values obtained from logistic analysis of the curves shown in Figure 3; (b) Dose-ratios derived from Gaddum analysis of EC_{50}* and IC_{50}* values obtained from the curves shown in Figure 5, as described in Methods; (c) Combination of (a) and (b). The lines show linear regression, with Schild slopes unconstrained (solid) or fixed at 1 (dotted).

bolic, otherwise the antagonist slope will be similar to the agonist slope but will also depend on the fixed agonist concentration. The logistic slopes of functional inhibition curves by themselves therefore provide no information as to e.g. the presence of multiple receptors, unless the agonist curve has a slope of 1: the Schild slope factor obtained by direct fitting to the Waud equation may be more informative.

Experimental

The presence of GDP was required to reveal an agonist induced increase in [^{35}S]-GPTγS binding mediated by m2 and m4 receptors, and improved the signal mediated by m1 and m3 receptors (Hilf *et al.*, 1989; Farries, unpublished observations). The optimal GDP concentration differed at different subtypes (Lazareno *et al.*, 1993; Farries, unpublished), here, 10^{-7} M (Experiment 1) and 3×10^{-7} (Experiment 2) was used with m1 and m3 receptors, and 10^{-6} M was used with m2 and m4 receptors.

It was found necessary to pre-equilibrate the membranes with both agonist and antagonist before initiating the [^{35}S]-GPTγS binding, since preincubation with pirenzepine alone led to concentration-dependent decreases in the agonist E_{max} at m1–m4 receptor subtypes (data not shown). Preincubation with ACh at m1 or m4 receptors did not reduce either the potency of ACh or its E_{max} (data not shown).

Experiment 1 ACh activation curves were constructed at m1–m4 receptors alone and in the presence of three concentrations of pirenzepine. The greater potency of ACh at m2 and m4 receptors allowed a wider range of pirenzepine concentrations than at m1 and m3 receptors.

Pirenzepine did not significantly affect basal activity, E_{max} or slope. Direct fitting of the data to the Waud equation revealed that the Schild slopes were not significantly different from 1, and Figure 3 shows the data with fits constrained to Schild slopes of 1. The parameter estimates are shown in Table 1.

Figure 4a shows conventional Schild plots derived from EC_{50} values obtained by logistic analyses in which the basal, E_{max} and slope parameter estimates were shared between the

Subtype/ Symbol	(▽)	(▼)	(□)	(■)
m1	0	3×10^{-8}	10^{-7}	3×10^{-7}
m2	0	3×10^{-7}	3×10^{-6}	3×10^{-5}
m3	0	3×10^{-7}	10^{-6}	3×10^{-6}
m4	0	10^{-7}	10^{-6}	10^{-5}

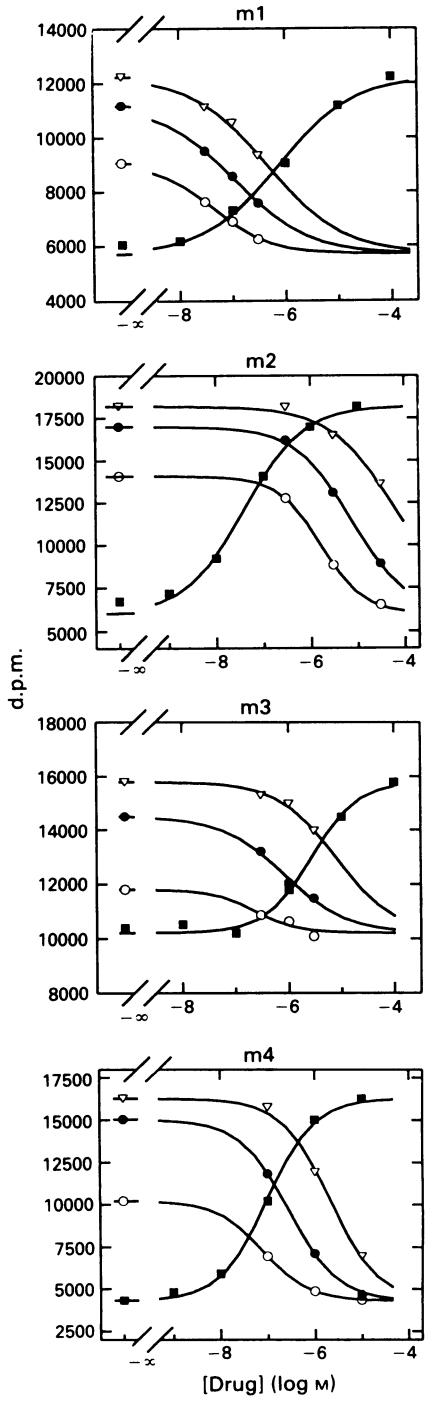

Figure 5 Inhibition curves of pirenzepine in the presence of different fixed acetylcholine (ACh) concentrations (○, ● and ▽), constructed from part of the data shown in Figure 3, together with control acetylcholine curves (■).

curves. The Schild slopes were not significantly different from 1 and the pK_b values were very similar to those obtained by direct fitting to the Waud equation (Table 1).

Figure 5 shows inhibition curves constructed from parts of the complete data set. Each inhibition curve was analysed together with the portion of the control activation curve up to the fixed agonist concentration [A], with common defined basal and maximum parameters, to yield EC_{50}* and IC_{50}* values. The dose ratios ([A]/EC_{50}*) and corresponding IC_{50}* values are shown as Schild plots in Figure 4b, and Schild plots combining the data from the activation and inhibition curves are shown in Figure 4c. The results (Figures 4b and 4c, Table 1) show that in all cases the Schild plots were linear with slopes close to 1. This reflects the fact that the pK_b estimates from the inhibition curves were close to the 'true'

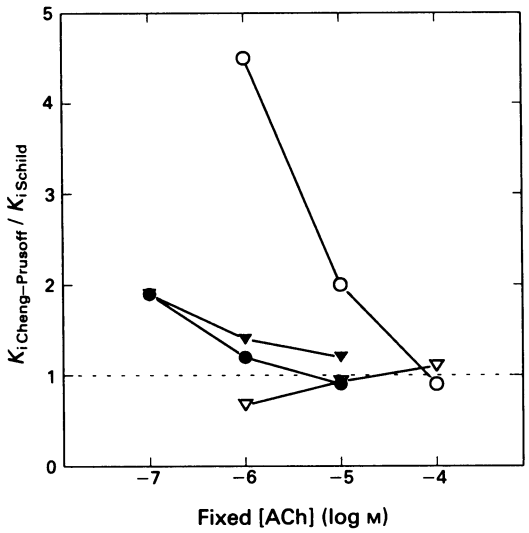

Figure 6 Error caused by using the Cheng-Prusoff equation to derive pirenzepine affinity estimates from the inhibition curves shown in Figure 5, at m1 (○), m2 (●), m3 (▽) and m4 (▼) receptors. The 'true' affinity was taken to be that derived from the direct fit to the Waud equation with Schild slope fixed at 1.

pK_b and were not correlated with the fixed agonist concentrations (data not shown). These results also show that the dose-ratios derived from the inhibition curves complement those obtained from activation curves to provide, in the case of m2 and m4 receptors, more detailed Schild plots over a wider range of antagonist concentrations (both those chosen by the experimenter and those estimated as IC_{50} values from inhibition curves).

Figure 6 shows the deviation of the Cheng-Prusoff estimates of pirenzepine dissociation constant from the 'true'

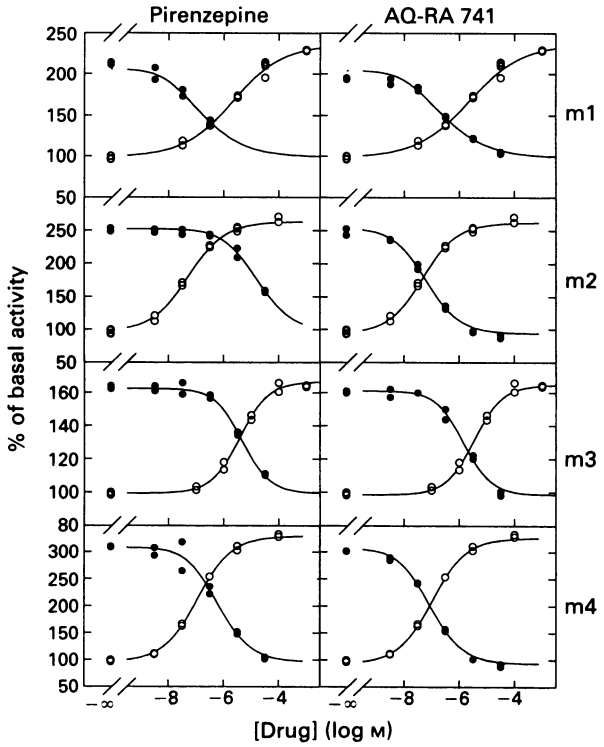

Figure 7 Experiment 2: acetylcholne activation curve for stimulation of GTPγS binding via m1–m4 muscarinic receptors (○) and inhibition curves for pirenzepine and AQ-RA 741 in the presence of a fixed acetylcholine concentration (●). The data are individual replicates expressed as % of mean basal activity. The lines show the direct fit of each activation + inhibition curve data set to the Waud equation with Schild slope fixed at 1.

values obtained from direct fitting to the Waud equation. The deviations are quite consistent with those derived theoretically (see Appendix and Figure 1) being minimal at the highest fixed agonist concentrations and at the m3 receptor, where the ACh curves had slopes close to 1.

Experiment 2 At each muscarinic receptor subtype, a full ACh activation curve was constructed, together with titrations of pirenzepine and AQ-RA 741 alone and in the presence of a fixed submaximal ACh concentration. In some cases the antagonists alone seemed slightly to inhibit basal activity (data not shown) with the largest apparent effect, 5–10% inhibition, seen with AQ-RA 741 at m4 receptors. These effects were too small and inconsistent to quantify and were not considered in subsequent analyses.

Figure 7 shows each inhibition curve and its corresponding activation curve fitted directly to the Waud equation with Schild slopes constrainted to 1. The unconstrained Schild slopes were not significantly different from 1 (Table 2), in marked contrast to the flat logistic slopes of the inhibition curves which are completely in agreement with the theoretical predictions shown in Figure 2.

Each inhibition curve was analysed together with the portion of the corresponding activation curve up to the fixed agonist concentration with basal and 'maximum' parameters constrained to be shared, to yield EC_{50}* and IC_{50}* values. pK_b estimates derived with the equation $pK_b = \log(IC_{50}^*/([A]/[EC_{50}^*]-1))$ were very close to the estimates obtained from the direct fit (Table 2).

The values obtained for pirenzepine in this experiment using single inhibition curves were almost identical to those obtained in Experiment 1 from full Schild analysis, and the affinity estimates for both antagonists are consistent with previously reported values (Lazareno *et al.*, 1990; Dorje *et al.*, 1991).

Discussion

Schild analysis is among the most robust and theoretically valid techniques in pharmacology (Colquhoun, 1987). Its robustness derives, in part, from the fact that the analysis is in two parts. The first part considers a family of two or more agonist titrations and allows the assumptions of parallel concentration-effect curves and constant E_{max} (both necessary if the antagonist is acting as a simple competitive inhibitor) to be assessed empirically. The second part considers dose-ratios derived from the first part, and tests the assumption that log(dose-ratio − 1) is a linear function of log antagonist concentration with slope of unity, again necessary if the antagonist is acting as a simple competitive inhibitor. As well as testing some of the underlying assumptions of the analysis, the two-stage nature of conventional Schild analysis allows the estimation of antagonist affinities from responses with which, for practical reasons, only a single dose-ratio estimate can be made in each experiment.

Conventional Schild analysis does have drawbacks, however, the most obvious of which is inefficiency: the analyses in Experiment 1 above each contained 56 data points and yet the conventional pA_2 estimate was derived from only three points. The range of antagonist concentrations which can be studied may also be limited by the potency of the agonist and hence the largest dose-ratio which can be measured without encountering nonspecific effects of high agonist concentrations.

In biochemical, and some whole-tissue, experiments it is often practically feasible to assess antagonist affinity by titrating the antagonist against a fixed concentration of agonist. Such a design allows efficient study of a large range of antagonist concentrations and is therefore widely used. The correct design and analysis of such experiments has, however, received little attention.

Binding experiments often use an inhibition curve design, and in principle the Cheng-Prusoff correction is a valid method for obtaining affinity estimates (though there are pitfalls in such experiments, and enhanced methods to account for them, see e.g. Munson & Rodbard, 1988). McKinney *et al.* (1991) have shown that the Cheng-Prusoff correction is valid in functional experiments where the agonist curve has a slope of 1, a conclusion with which we agree. In contrast, Eglen & Whiting (1989) claim to have demonstrated, both empirically and theoretically, that application of the Cheng-Prusoff equation is invalid at high agonist concentrations. This conclusion is surprising, since at high agonist concentrations the Cheng-Prusoff equation is almost identical to the null method equation of Lazareno & Roberts (1987, see Methods above). In fact, Eglen & Whiting showed only that application of the equation to their data yielded discrepant results, they did not demonstrate that the error lay in the application of the equation rather than in the data: in fact, application of a null method of analysis to their inhibition data would have given the same discrepant results. Leff & Dougall (1993) have recently derived a theoretically valid form of the Cheng-Prusoff equation which takes account of the slope of the agonist curve.

From our theoretical consideration, the following conclusions can be made regarding the applicability of the Cheng-Prusoff equation to those functional preparations which can be described by the Operational Model of Black & Leff (1983): (1) the equation is valid for rectangular hyperbolic E/[A] functions; (2) the equation is not valid for other E/[A] functions except when high fixed agonist concentrations are used (at least 10 fold greater than EC_{50}); (3) the equation gives the greatest error when concentrations between $EC_{50}/10$ and EC_{50} are used as fixed agonist concentrations; (4) the Hill slope of the E/[A] function is the major determinant of the size of the error – the equation gives increasing error as the slope deviates from 1, but for slopes between 0.7 and 1.2 the error will not exceed about 4 fold; (5) for a particular Hill slope, the error will increase with decreasing receptor reserve. The empirical results shown in Figure 6 are consistent with these conclusions, as are results obtained from a larger population of data (Lazareno & Birdsall, 1993).

Although use of the Cheng-Prusoff correction is invalid, there is a valid method for analysing a single inhibition curve, together with an activation curve, to provide a dose-ratio from which a valid pK_b can be estimated (Lazareno & Roberts, 1987, see Methods above). We found that these pK_b estimates were in excellent agreement with pA_2 values measured with full Schild analysis. The logistic slope of the inhibition curve, however, cannot be interpreted directly since it is strongly determined by the slope of the agonist curve. It is, nevertheless, possible to estimate the Schild slope from an inhibition curve. In principle, each point on an inhibition curve could be converted to a dose-ratio and the data could be treated as a Schild plot. We have found, however, that this treatment magnifies small errors at the top and bottom of the curve and does not provide reliable data. The solution is to use non-linear regression analysis to fit the combined inhibition and activation curves directly to the Schild model using the Waud equation. This provides reliable estimates of both the K_b and the Schild slope, and thus this design and analysis allows a test of one of the underlying assumptions of Schild analysis, namely that competitive antagonists have Schild slopes of 1. It is important to note that the slope of the inhibition curve can *only* be interpreted by using this analysis in conjunction with the activation curve.

Since a single inhibition curve can be used to provide an accurate estimate of K_b, it follows that a family of inhibition curves, with different fixed agonist concentrations, can be used to construct a Schild plot. Pöch *et al.* (1992) constructed families of activation curves in the normal Schild design and then extracted inhibition curves from their data, as we did in Experiment 1 above. They also showed that their 'alternative Schild plots', using dose-ratios derived from the inhibition curves, gave essentially the same results as the 'conventional'

Schild plots. Their analysis differs from our form of Gaddum analysis only in that they used the parameters of the logistic fit to the control activation curve to estimate the concentration of agonist alone giving the effect of 50% the fixed agonist concentration. Our results are completely consistent with those of Pöch *et al.* (1992), and demonstrate that dose-ratios obtained from inhibition curves have equal status with those obtained from activation curves. It therefore makes sense, if the design of the experiment allows, to consider the data from a family of activation curves also as a family of inhibition curves and to combine the dose ratios from both types of curves into a single Schild plot. As shown above, this can double the number of points on the Schild plot and provide more precision and a wider range of antagonist concentrations. Much attention has been given to the significance and analysis of Schild plots which are not linear or have slopes different from 1 (Kenakin, 1982). The use of inhibition curves to obtain more detailed and extensive Schild plots may therefore be of practical value.

A way to enhance further the power and efficiency of the full Schild analysis is to fit the entire data set directly to the Schild model with the Waud (1975, 1976) equation using non-linear regression analysis. This procedure uses all the data, rather than just a few dose-ratio values, to estimate the pA_2 and Schild slope, and it therefore provides better estimates of these parameters. This in turn means that fewer data points are required for reliable results.

In conclusion, antagonist affinities can be estimated from functional inhibition curves either by a null method of dose-ratio analysis or by a direct fit to the Schild model with the Waud equation. Use of the Cheng-Prusoff equation is usually invalid and inaccurate, and always unnecessary.

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Appendix

Theoretical assessment of the validity of application of the Cheng-Prusoff equation to functional experiments, using the Operational Model of agonism (Black & Leff, 1983)

The operational model starts with the same basic assumptions as the traditional null methods, that agonists and competitive antagonists interact at the receptor according to the Law of Mass Action and that the response is a function of agonist receptor occupancy at equilibrium. In addition, the operational model assumes explicitly that the function relating occupancy to response (the 'transducer function') is a logistic function. While such a model cannot be universally true, it is sufficiently general to be of interest, and has been found to generate consistent results when applied to pharmacological data (Leff, 1988).

Receptor occupancy by agonist alone is given by

$$[AR] = \frac{R_0 \cdot [A]}{[A] + K_A} \quad (1)$$

where [AR] = concentration of agonist-occupied receptors, R_0 = total receptor concentration, [A] = agonist concentration, K_A = agonist dissociation constant.

Receptor occupancy by agonist in the presence of competitive antagonist is given by

$$[AR] = \frac{R_0 \cdot [A]}{[A] + K_A \cdot \left(1 + \frac{[I]}{K_I}\right)} \quad (2)$$

where [I] = antagonist concentration, K_i = antagonist dissociation constant.

Response is assumed to be a logistic function of agonist occupancy

$$E = \frac{E_m \cdot [AR]^n}{K_E{}^n + [AR]^n} \quad (3)$$

where E = the response, E_{max} = the maximum possible response of the tissue, K_E = the concentration of agonist-occupied receptors required to produce a response of $E_m/2$, n = the slope factor of the logistic transducer function.

A useful quantity, τ (the 'transducer ratio'), is defined as R_0/K_E, and is a function of the efficacy of the agonist.

Substituting (1) into (3) we get

$$E = \frac{E_m \cdot \tau^n \cdot [A]^n}{([A] + K_A)^n + \tau^n \cdot [A]^n} \quad (4)$$

and

$$[A_{50}] = \frac{K_A}{(2 + \tau^n)^{\frac{1}{n}} - 1} \quad (5)$$

where $[A_{50}]$ = the agonist concentration giving half its own maximum response.

When the concentration-effect curve is rectangular hyperbolic, the transducer function has a slope of 1

$$E = \frac{E_m \cdot \tau \cdot [A]}{K_A + (1 + \tau) \cdot [A]} \quad (6)$$

and

$$[A_{50}] = \frac{K_A}{1 + \tau} \quad (7)$$

In the presence of competitive antagonist we substitute (2) into (3) and obtain

$$E = \frac{E_m \cdot \tau^n \cdot [A]^n}{([A] + K_A \cdot (1 + \frac{[I]}{K_I}))^n + \tau^n \cdot [A]^n} \quad (8)$$

and, if the transducer function has a slope of 1,

$$E = \frac{E_m \cdot \tau \cdot [A]}{[A] \cdot (1 + \tau) + K_A \cdot (1 + \frac{[I]}{K_I})} \quad (9)$$

Consider first the simple case, where the slope, n, of the transducer function is 1 (which will result in the concentration-effect curve also having a slope of 1).

If $[I_{50}]$ is the antagonist concentration which inhibits the effect of a particular agonist concentration by 50%, then, from (6) and (9),

$$\frac{2 \cdot E_m \cdot \tau \cdot [A]}{[A] \cdot (1 + \tau) + K_A \cdot (1 + \frac{[I_{50}]}{K_I})} = \frac{E_m \cdot \tau \cdot [A]}{[A] \cdot (1 + \tau) + K_A}$$

and

$$K_I = \frac{[I_{50}]}{[A] \cdot \frac{1 + \tau}{K_A} + 1} \quad (10)$$

Substituting the equation for $[A_{50}]$ (7)

$$K_I = \frac{[I_{50}]}{\frac{[A]}{[A_{50}]} + 1} \quad (11)$$

which is the Cheng-Prusoff equation. So, if the concentration-effect curve has a slope of 1 the Cheng-Prusoff equation gives the correct answer for any concentration of agonist (this conclusion has recently been derived in a different way by KcKinney *et al.* (1991)).

Consider now the more general case. From (4) and (8)

$$\frac{2 \cdot E_m \cdot \tau^n \cdot [A]^n}{([A] + K_A.(1 + \frac{[I_{50}]}{K_I}))^n + \tau^n \cdot [A]^n} = \frac{E_m \cdot \tau^n \cdot [A]^n}{(K_A + [A])^n + \tau^n.[A]^n}$$

$$([A] + K_A + K_A.\frac{[I_{50}]}{K_I})^n = 2.(K_A + [A])^n + \tau^n.[A]^n \quad (12)$$

With very large [A], ($[A] >> K_A$)

$$[A] + K_A.\frac{[I_{50}]}{K_I} = [A].\ (2 + \tau^n)^{\frac{1}{n}} \quad (13)$$

Substituting for K_A in (5) gives

$$K_I = \frac{[I_{50}]}{\frac{[A]}{[A_{50}]}} \quad (14)$$

which is the same as the Cheng-Prusoff equation at large [A], so with high agonist concentrations the Cheng-Prusoff equation gives the correct result.

With very small [A], ([A] → 0)

$$(K_A.\ (1 + \frac{[I_{50}]}{K_I}))^n = 2.K_A^n \quad (15)$$

$$K_I = \frac{[I_{50}]}{2^{\frac{1}{n}} - 1} \quad (16)$$

If K_{CP} is the estimate of K_i obtained with the Cheng-Prusoff equation, then as [A] → 0, $K_{CP} = [I_{50}]$, and

$$\frac{K_{CP}}{K_I} = 2^{\frac{1}{n}} - 1 \quad (17)$$

i.e. at very low agonist concentrations K_{CP} will overestimate K_i if $n < 1$, and will underestimate K_i if $n > 1$.

In general, from (12), the antagonist $[I_{50}]$ is related to the fixed agonist [A] by

$$[I_{50}] = \frac{K_I}{K_A} \cdot ((2.(K_A + [A])^n + \tau^n.[A]^n)^{\frac{1}{n}} - [A] - K_A) \quad (18)$$

The Cheng-Prusoff estimate of K_i (K_{CP}) can then be calculated using the formula for $[A_{50}]$ (5) and $[I_{50}]$ (18).

Hill slopes of agonist E/[A] curves

The 'Hill slopes' quoted in Figure 1 are necessarily approximate because, for n = 1, the E/[A] curve is not a logistic function (Black *et al.*, 1985). The deviation from a logistic function is small, however, so it may reasonably be assumed that the gradient of the midpoint on the E/[A] curve is equal to the midpoint gradient of the logistic function best describing the E/[A] curve. The midpoint gradient of a normalised E/log[A] curve generated by the operational model is (Black *et al.*, 1985)

$$G_{\frac{1}{2}(operational)} = \frac{0.576.n.(2 + \tau^n).((2 + \tau^n)^{1/n} - 1}{(2 + \tau^n)^{1/n}.(1 + \tau^n)} \quad (19)$$

and the midpoint gradient of a logistic function is

$$G_{\frac{1}{2}(logistic)} = 0.576 \cdot \text{Hill slope} \quad (20)$$

so the Hill slope is estimated as

$$\text{Hill slope} = \frac{n.(2 + \tau^n).((2 + \tau^n)^{1/n} - 1)}{(2 + \tau^n)^{1/n}.(1 + \tau^n)} \quad (21)$$

Within the range of values used in Figure 1 the deviation between these Hill slope estimates and slope factors obtained from logistic analysis of simulated E/[A] curves was about 0.03 or less. The value of n corresponding to a given Hill slope was estimated from (21) with an iterative procedure.

When τ is large, Hill slope $\approx$ n, but when τ is small the Hill slope tends towards 1 (Black *et al.*, 1985) so, for a given Hill slope, n $\approx$ Hill slope when τ is big, and n increasingly deviates from 1 as τ becomes smaller. The size of the deviation of the Cheng-Prusoff estimate as [A] → 0 depends only on n and is independent of τ (17), and it increases as n deviates from 1. For a given Hill slope, however, the deviation of the Cheng-Prusoff estimate is inversely related to τ, i.e. the deviation is large when receptor reserve is small and Hill slope $\neq$ 1.

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