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Antagonist inhibition curves and the measurement of dissociation constants

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1 Experiments carried out on guinea-pig isolated ileum with carbachol as agonist and diphenyl-acetoxyethyl- dimethyl-ethyl- ammonium (DADMEA) bromide as antagonist gave results which fit the theoretical relation between fractional inhibition (Q) of the effects of an agonist ([A]) and the concentration of a competitive antagonist ([B]): this also involves the Hill coefficient (logistic slope factor, P) for the agonist concentration-response curve and the degree of agonist stimulation, $[A]/[A]_{50}$, where $[A]_{50}$ produces a half-maximum response.

2 Values of IC_{50} and an exponent, P′, can be obtained by fitting Q to [B] using a logistic approximation to the relation. Both P′ and IC_{50} should be greater with higher agonist stimulation but the increase in P′ may be masked by errors in extreme values of Q. Estimates of IC_{50}, however, invariably increased with higher agonist stimulation but with a steep concentration-response curve ($P>1$) and low agonist stimulation ($[A]/[A]_{50}<1$), IC_{50} can be less than K_D.

3 K_D was calculated from the results in three ways: (i) by a least-squares fit of Q to [B] using the values of P and $[A]/[A]_{50}$ calculated from the control concentration-response curve; (ii) from the value of IC_{50} for each line and the values of P and $[A]/[A]_{50}$ and (iii) by using the agonist concentration-response curve to calculate the dose-ratio and estimate of K_D for each response in the presence of the antagonist. The methods gave similar results (nM: 11 experiments), 12.4 ± 1.1 (i), 11.7 ± 0.9 (ii), 14.8 ± 1.6 (iii) but there are advantages in using methods (i) or (ii) rather than (iii).

4 The method by which K_D is calculated is less important than the experimental design: the plan used in this work, with alternative small and large responses from the tissue, is very suitable for estimating K_D with low concentrations of antagonists and small dose-ratios. Although it is not a sensitive test for competitive behaviour because only a small range of concentrations of antagonist is tested, the estimate of affinity should be free from complications involved in the use of higher concentrations of antagonist (and agonist) and the nature of the antagonism can always be checked by doing further experiments in the presence of a known competitive antagonist.

Keywords: Antagonist dissociation constants; K_D; pA_2; dose-ratios; inhibition curves; IC_{50}; competitive antagonists; degree of agonist stimulation; Hill coefficient; logistic slope factor

Introduction

Because the effect of an antagonist can be seen only when an agonist is present, it is natural to express what it does as a percentage reduction in the effect of the agonist. If the reduction is plotted against the concentration of antagonist on a logarithmic scale, the curve is sigmoid and the effect of the antagonist can be expressed as the concentration producing 50% inhibition, IC_{50}. This is not constant, however, and depends on the concentration of agonist. For example, in experiments in which the binding of a labelled ligand is competitively antagonized by an unlabelled one, the IC_{50} for the unlabelled compound is greater than its equilibrium dissociation constant, K_D:

$$IC_{50} = K_D(1 + [A]/K_A)$$

where [A] is the concentration of the labelled ligand and K_A is its equilibrium dissociation constant (Cheng & Prusoff, 1973).

In experiments in which an antagonist affects the response produced by an agonist on a tissue, the relation between agonist concentration and response is complex: the binding may follow the law of mass action (Gaddum, 1937) but the response curve can be steeper than the binding curve, indicating non-linear amplification of the signal. To measure the effect on an antagonist in such experiments Schild (1947, 1949) developed the idea of expressing the antagonist's effect in terms of a 'dose-ratio': the agonist was increased to overcome the effects of the antagonist and the dose-ratio was calculated by dividing the concentration (dose) of agonist producing the response in the presence of the antagonist by the concentration (dose) producing the same response when it was absent. If the antagonism is competitive, the relation between dose-ratio (DR) and antagonist concentration, [B], is given by the Gaddum-Schild equation:

$$DR = 1 + [B]/K_D \qquad (1)$$

where K_D is the antagonist dissociation constant. Measurements of dose-ratio are commonly used to calculate an antagonist equilibrium constant and Schild devised the term pA_X to describe log(1/[B]) for a dose-ratio of X, so $pA_2 = -\log(K_D)$. It has long been realised, however, that the errors in estimates of K_D or log (K_D) are greater with small dose-ratios than with large (e.g. Edinburgh Staff, 1968): the error in DR is likely to be proportional to DR but K_D is calculated from $(DR-1)$. In particular, estimates of K_D based on a dose-ratio of 2 will be much less accurate than those based on a dose-ratio of 10 or more.

Recently there has been considerable interest in the relation between inhibition and dose-ratio (Craig, 1993; Leff & Dougall, 1993; Lazareno & Birdsall, 1993a,b; Barlow, 1995). If Q is the fractional inhibition and DR is the dose-ratio, then:

$$Q = \frac{(DR^P - 1)}{DR^P + ([A]/[A_{50}])^P} \qquad (2)$$

where $[A]/[A]_{50}$ is the concentration of agonist used divided by

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the concentration producing a half-maximal response: it indicates the position on the agonist concentration-response curve and has been called the degree of agonist stimulation: P is the exponent (Hill coefficient, logistic slope factor) for the agonist concentration-response curve.

$$Y = \frac{M\ [A]^P}{[A]^P + [A]_{50}{}^P} \quad (3)$$

where Y is the response, M is the maximum response and $[A]_{50}$ is the concentration producing a half-maximum response.

The relation between fractional inhibition, Q, and antagonist concentration, [B], is obtained by combining equation 2 with equation 1 and is illustrated in Figure 1. With high agonist stimulation the complex relation approximates to:

$$Q = \frac{([B]/K_D)^P}{([B]/K_D)^P + ([A]/[A]_{50})^P} \quad (4)$$

which is logistic with the exponent P having the same value as the Hill coefficient for the agonist (Barlow, 1995).

With low agonist stimulation the relation can be calculated by using only the first terms in the binomial expansion of $(1+[B]/K_D)^P$, i.e. [B] is small and $[B]^2$ etc. can be neglected. This gives

$$Q = \frac{[B]}{K_D/P + [B]}$$

i.e. inhibition is determined only by the binding of the antagonist.

The logistic equation:

$$Q = \frac{[B]^{P'}}{[B]^{P'} + IC_{50}{}^{P'}} \quad (5)$$

can therefore be taken as an approximation to the full relation obtained by combining equations 1 and 2, and can be used empirically when P for the agonist and the degree of agonist stimulation, $[A]/[A]_{50}$, are unknown, such as occurs when it is released physiologically.

The value of IC_{50} obtained is related to K_D by the equation:

$$IC_{50}/K_D = [2 + ([A]/[A]_{50})^P]^{1/P} - 1 \quad (6)$$

When P = 1, this is the same as the Cheng-Prusoff equation and IC_{50} is always greater than K_D, but when the degree of agonist stimulation is small and P is greater than one, IC_{50} should be less than K_D.

This paper describes attempts to produce experimentally the curves shown in Figure 1 and to investigate the effects of the degree of agonist stimulation on P′ and IC_{50}. From the results it became apparent that the experimental design was particularly appropriate to the measurement of K_D using concentrations of antagonist which produced only small dose-ratios and different methods of calculating K_D have been compared. The guinea-pig isolated ileum was used with carbachol as agonist and diphenylacetoxyethyl-dimethyl-ethyl-ammonium (DADMEA) bromide as antagonist: this is known to act competitively over a range of concentrations (Barlow *et al*., 1963; Abramson *et al*., 1969).

Methods

Guinea-pig isolated ileum

Short lengths of ileum (about 15 mm) from male Dunkin-Hartley guinea-pigs (500 – 700 g) were set up as described by Edinburgh Staff (1968) in Krebs solution containing hexamethonium bromide (100 μM), aerated with a mixture of oxygen (95%) and carbon dioxide (5%). The volume of the bath was about 8 ml and contractions of the longitudinal muscle were recorded isotonically with a load of about 0.5 g. The temperature was 37°C.

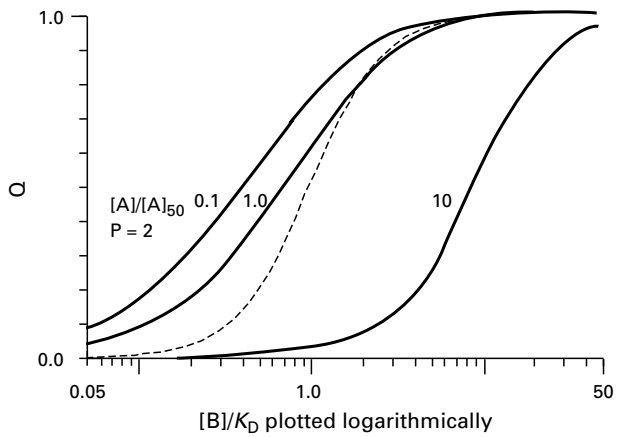

Figure 1 Relation between inhibition (Q) and antagonist concentration as a fraction of K_D (i.e. $[B]/K_D$) for a competitive antagonist obtained by combining equations 1 and 2. The agonist has P (Hill coefficient, logistic slope factor) = 2 and the degree of agonist stimulation, from left to right, $[A]/[A]_{50} = 0.01$ and 0.1 (superimposed), 1 and 10. The broken line shows the relation given by equation 5 with $P' = 2$ and $IC_{50}/K_D = 1$.

The agonist, carbachol, was allowed to act for 30 s and added once every 2.5 min by computer-operated relays. At the start of the experiment responses were obtained to a wide range of concentrations of agonist to obtain some idea of the maximum. Two concentrations were then selected which gave responses respectively less than 50% ('low') and greater than 50% ('high'). These were given alternatively until they were regular and are referred to as the control (low or high) responses. The preparation was then exposed to the lowest concentration of antagonist. When the responses were regular the tissue was exposed to the next concentration of antagonist and so on. At the start of the work the concentrations treated were 8, 12, 18, 24 and 30 nM but these were later altered so as to try to produce a suitable range of inhibition (5% to 95%) with both low and high agonist stimulation. The inhibition produced by each concentration of antagonist was calculated from the responses (usually 4 sets) in the presence of the antagonist and the mean of the control responses (usually 4 sets).

Compounds

Carbachol and hexamethonium bromide were obtained from Sigma. The sample of diphenylacetoxyethyl dimethyl-ethylammonium bromide was that used by Abramson *et al*. (1969): it was crystalline and had been stored away from light.

Data analysis

All the responses in the absence of the antagonist were fitted to the Hill equation (3) to obtain estimates of P and $[A]_{50}$ and hence calculate the degree of agonist stimulation, $[A]/[A]_{50}$, for the low and high agonist responses. The values of inhibition, Q, were then fitted by least-squares to the antagonist concentration, [B], using the combination of equations 1 and 2: the remaining unknown, K_D, was altered stepwise from an initial estimate until the improvement in fit was considered negligible. This procedure was used to fit both lines obtained from any one preparation as well as for separate results for low and high agonist stimulation and is referred to as method (i) for the calculation of K_D.

The values of Q were fitted by least-squares to values of [B] using equation 5 and give estimates of IC_{50} and P′ for low and high agonist stimulation. These values, together with $[A]/[A]_{50}$ and P from the agonist concentration-response curve, were then used to calculate K_D from equation 6: this is method (ii).

Method (iii) for calculating K_D involved obtaining the matching concentration of agonist (from the agonist concentration-response curve) for the mean response in the presence of a particular concentration of the antagonist. This was converted into the dose-ratio and hence into an estimate of K_D for that concentration, using equation 1.

The programmes used for the fit to equations 3 and 5 were described by Barlow (1983): these give estimates of the standard errors of the parameters calculated, based on the within-experiment variance. Copies of those used for method (1) can be obtained from the authors. In all of them the degree of fit is indicated by the sum of the squares of the deviations expressed as a percentage of the sum of the squares of the observations (Sd^2 as %: comparable to the coefficient of variation): the smaller this is, the better the fit.

Results

Two sets of experiments were carried out and details of the first are shown in Table 1. The variation in the sensitivity of the preparations can be seen from the range of the values of P (1.05–3.1) and of $[A]_{50}$ (0.08–0.19 μM) for the agonist concentration-response curve. The degree of agonist stimulation, $[A]/[A]_{50}$, was usually less than 1 for the low responses and greater than 1 for the high responses and the value (crit.) for which IC_{50} should equal K_D was calculated from equation 6. If the results are consistent with competition, the values of K_D should be the same for low and high stimulation. When they are, the fit of Q to [B] using both lines provides experimental support (Figure 2) for the theoretical lines illustrated in Figure 1: these experiments have low values of Sd^2, e.g. $<1\%$.

In all experiments shown in Table 1 IC_{50} was greater for the high responses than for the low, for which the probability is 1 in $2^{10}=1024$. In all except experiment 5, the values of P′ were less than P for the agonist controls but P′ is only greater for the inhibition of higher responses in 7 out of 10 experiments (probability = 0.12). It is clear that there are more errors in some experiments than in others: in experiments 1, 2, 3, 5, 6 and 7 the fitted value of IC_{50} lies outside the range of concentrations tested. In experiments 3, 4, 5 and 6 some of the responses gave dose-ratios <1 because the tissue had become more sensitive during the experiment.

The effects of errors on the least-squares fit of results to equation 5 was examined by a method used for other relations (Barlow, 1993), in which a set of theoretical values of B and Q is taken and the effect of increasing each point in turn from $Q-0.05$ to $Q+0.05$ is observed. The results are shown in Figure 3 and indicate that values of P′ are particularly sensitive to errors in very small or very large values of Q. If the inhibition with low concentrations is overestimated, P′ is underestimated and this may account for some of the instances where $P'<1$. It may also explain why the increase in slope with increasing agonist stimulation may not be seen: the values of P′ should lie between 1 and P, a narrow interval, and the increase could be masked by errors in low values of Q.

In contrast to the effects on P′, the estimate of IC_{50} is less affected by errors in individual points and the biggest effects are produced by errors in points in the middle range, which are anyway more likely to be accurate than those at the extremes. A test can be made by comparing the degree of agonist stimulation with the critical value and IC_{50} with K_D, using the value for the appropriate line calculated by method (iii). If P = 1, IC_{50} must be greater than K_D and in experiment 1, which has the unusually low value P = 1.05, IC_{50} for the low degree of agonist stimulation is actually lower than K_D whereas for the high degree of stimulation $IC_{50}>K_D$: in experiment 2, however, the results for both lines are qualitatively as expected. In all 16 out of 20 results are as expected (probability = 0.005).

A graphical check which can be made by calculating IC_{50}/K_D for each line and plotting it against $[2+([A]/[A]_{50})^P]^{1/P}$. This should give a straight line with a slope of 1 and a constant of -1: if the value for high agonist stimulation in experiment 7 (with $IC_{50}=109$, well outside the range of concentrations tested) is excluded, a least-squares fit (Figure 4a) gives: slope $=0.982\pm0.117$ (s.e., 17 d.f.), constant -1.19 ± 0.31.

Table 1 Results on guinea-pig ileum with carbachol as agonist showing the concentrations of antagonist tested, the values of $[A]_{50}$ and P for the controls, $[A]/[A]_{50}$ for the low and high responses and the value (crit.) at which IC_{50} should be the same as K_D

Experiment	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*mean ±s.e.mean*	*1m*
Concs	8 12 18	8 12 18	2 4 8	2 4 8	2 8 16	2 8 16	3 8 16	3 8 16	3 8 16	3 8 16		
(nM)	24 30	24 30	18 24 30	18 24 30	24 30	24 30	25 40	25 40	25	25		
$[A]_{50}\mu M$	0.127	0.131	0.127	0.101	0.122	0.117	0.112	0.077	0.094	0.192		
P	1.05	1.99	1.86	1.54	1.65	2.06	1.29	1.81	1.45	3.08		
$[A]/[A]_{50}$												
low	0.630	0.611	0.787	0.987	0.820	0.853	0.446	1.039	0.850	1.042		
high	3.150	3.053	1.574	1.974	3.279	3.413	3.571	2.597	2.125	2.084		
crit	0.08	1.41	1.30	0.94	1.08	1.46	0.53	1.25	0.81	1.83		
IC_{50}	low 2.44*	5.54*	39.3	17.0	22.7	15.1	21.5	7.73	9.80	3.62		
	high 56.7*	19.7	41.6*	28.4	57.2*	35.6*	109*	19.6	16.2	11.5		
P′	low 0.80	1.49	0.77	1.22	1.68	0.82	0.81	0.90	1.20	1.19		
	high 0.69	1.78	1.01	1.25	1.77	2.00	0.56	1.39	0.78	1.48		
K_D												
(i)	low 2.32	9.22	45.7	16.8	29.3	26.0	22.2	10.6	9.62	9.45	18.1±4.08	13.9
	high 10.1	8.48	30.6	16.2	28.4	15.1	14.1	9.63	7.68	10.1	15.0±2.58	13.4
	mean 6.21	8.85	38.2	16.5	28.9	20.6	18.2	10.1	8.65	9.78	16.6±3.26	14.1
(ii)	low 1.63	10.2	57.3	16.5	27.2	24.1	22.8	9.96	9.48	8.06	18.7±5.00	13.3
	high 14.4	8.31	34.7	15.9	20.3	13.3	30.2	9.43	7.99	9.46	16.4±2.96	14.4
	mean 8.02	9.26	46.0	16.2	23.8	18.7	26.5	9.70	8.74	8.76	17.6±3.81	14.7
(iii)	low 2.80	8.19	87.7*	33.3**	32.6**	40.1	21.1	11.9	20.4	11.8	27.0±7.75	18.7
	high 12.2	8.90	27.5*	16.8*	34.5*	22.3**	11.9	12.6	7.42	8.46	16.3±2.86	14.3
	mean 7.50	8.55	57.6	25.1	33.6	31.2	15.4	12.3	13.9	10.1	21.5±4.98	17.4
Fit to 2 lines												
	5.36	8.68	38.4	16.5	29.1	21.2	18.9	10.0	8.60	9.88	16.7±3.33	14.0
	6.3%	0.1%	5.7%	0.6%	3.2%	4.7%	4.0%	0.8%	1.4%	0.7%		

Values of IC_{50} and P′ are calculated: (i) by a least-squares fit of each line separately; (ii) from the values of IC_{50} using equation 6; (iii) from the dose-ratios and antagonist concentrations for each line and finally from the fit of both lines, shown with Sd^2 as %. Asterisks mark estimates of IC_{50} which lie outside the range of concentrations tested or instances where the dose-ratio was less than 1. Mean values are shown (±s.e.mean): 1m indicates the logarithmic means, which show less scatter.

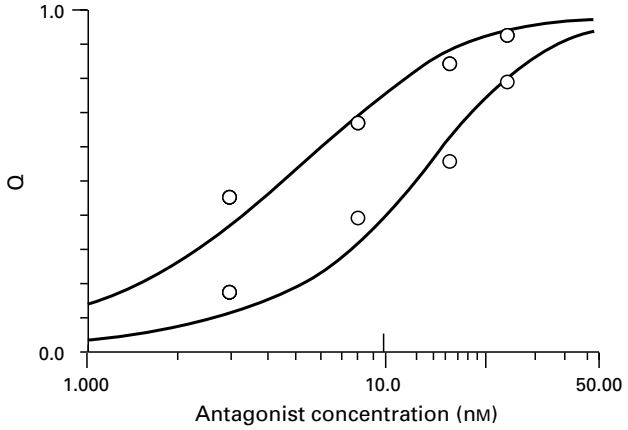

Figure 2 Inhibition plotted against antagonist concentration (experiment 10). The lines show the least-squares fit of both sets of results (method (i)), which gives $K_D = 9.88$, Sd^2 0.7%: $P = 3.08 \pm 0.96$ (s.e., 10 d.f.) for the agonist concentration-response curve and the estimates of P′ using equation 5 are 1.19 ± 0.14 (s.e., 2 d.f.) and 1.48 ± 0.25 (s.e., 2 d.f.) for $[A]/[A]_{50} = 1.04$ and 2.08, respectively.

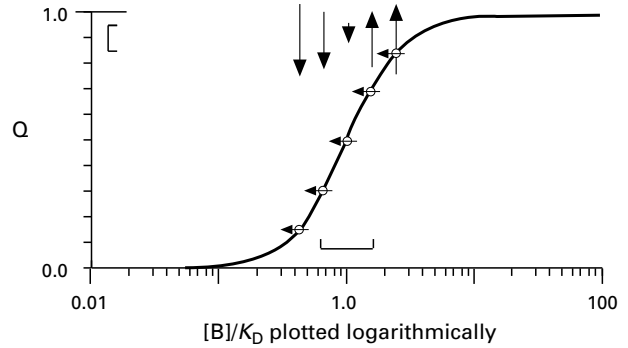

Figure 3 Effects of errors on estimates of P′ and IC_{50} obtained with theoretical data for equation 5 with $P' = 2$ and $IC_{50}/K_d = 1$. Each value of Q was altered in turn from $Q - 0.05$ to $Q + 0.05$: the vertical arrows show the effect on P′ and the horizontal arrows the effect on IC_{50} (the scales show 0.1 units). Note the profound effects on P′ of errors in points at either end and the small effects on IC_{50}.

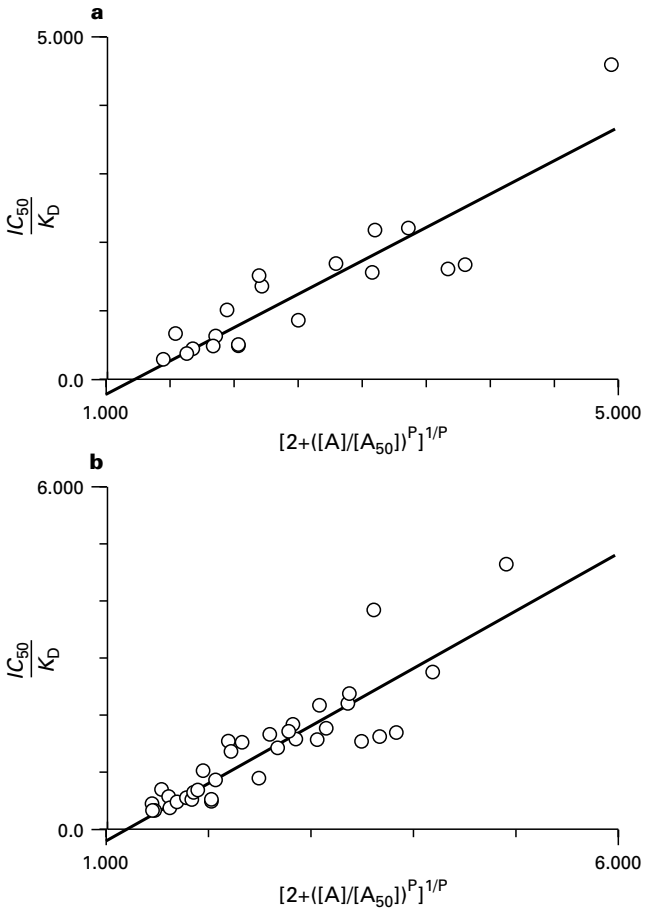

Figure 4 (a) Graph of IC_{50}/K_D against $[2 + ([A]/[A]_{50})^P]^{1/P}$ for the results in Table 1 (20 points): if the value for high agonist stimulation in experiment 7 (with $IC_{50} = 109$, well outside the range of concentrations tested) is excluded, a least-squares fit gives: slope ($\pm$s.e.) = 0.982 ($\pm$0.117); constant = -1.19 ($\pm$0.31). (b) Graph for all data excluding IC_{50} = 109 (i.e. 37 points): a least-squares fit gives: slope ($\pm$s.e.) = 0.998 ($\pm$0.084); constant = -1.18 ($\pm$0.22).

There is a big variation in the estimates of K_D but the differences between values for low and high agonist stimulation are not consistent, as they should be if the compound does not behave competitively. An analysis of variance gives $F = 0.68$ for the difference between methods and $F = 2.08$ for the difference between low and high agonist stimulation compared with 5.14 and 5.99 for the respective values for 5% probability and an analysis of variance based on ranks gives a probability $> 10\%$ for both factors. It seems appropriate to express the result of any one experiment as the mean of the values for low and high agonist stimulation and with method (i) the value obtained with the fit to two lines should be the same as the logarithmic mean of the values obtained with each separately.

Because there appeared to be experimental errors in so many of the results shown in Table 1, with only 4 having $sd^2 < 1\%$ using the fit to both lines and method (i), a second set of 9 experiments was carried out four months later. The range of concentrations of antagonist (usually 3–50 nM) was better chosen. In only 2 experiments did an estimate of IC_{50} lie outside the range of concentrations: 4 experiments had $Sd^2 < 1\%$ and in only 1 was $Sd^2 > 6\%$. The increase in P′ with agonist stimulation, however, was observed only in 5 experiments but if the results are included along with those shown in Figure 4a, the graphical check using IC_{50}/K_D (Figure 4b) gives slope = 0.998 ± 0.084 (s.e., 35 d.f.), constant = -1.18 ± 0.22. The results of the second set of experiments are summarized in Table 2A and the results for all experiments with $Sd^2 > 2.5\%$ are shown in Table 2B.

Discussion

This work shows that, with care, it is possible to obtain experimental results which fit the theoretical curves in Figure 1. The expected steepening of the antagonist inhibition curve (Q against [B]) is more difficult to observe because the changes in P′ are small and may be masked by errors associated with overestimates of the effects of low concentrations of antagonist: values of Q should be limited to the range 0.05 to 0.95. There are consistent effects on IC_{50}, however, which include many observations that although this is greater than K_D with high agonist stimulation, it is less than K_D with a steep agonist concentration-response curve and low agonist stimulation ($P > 1$ and $[A]/[A]_{50} < 1$).

There is close agreement between the mean estimates of K_D for the two sets of experiments (Table 2A) but there is considerable variation between individual experiments. Does this variation indicate that K_D is really different or that it appears to be different, perhaps because of the way in which it is calculated? When estimates obtained by the different methods are plotted against each other (Figure 5) the points lie fairly close to the line of identity. This is consistent with the lack of significant

Table 2 **A** Results in the second set (9 experiments) compared with those of the first set (10 experiments: shown in parentheses)

	Low	*High*	*Mean*	*2 lines*
Method (i)				
Mean K_D	16.7 (18.1)	14.1 (15.0)	15.4 (16.6)	14.9 (16.7)
s.e.mean	3.11 (4.08)	1.05 (2.58)	1.77 (3.26)	1.65 (3.33)
Logarithmic mean	14.5 (13.9)	13.7 (13.4)	14.5 (14.1)	14.1 (14.0)
Method (ii)				
mean K_D	14.7 (18.7)	13.6 (16.4)	14.1 (17.6)	
s.e.mean	2.67 (5.00)	0.86 (2.96)	1.44 (3.81)	
Logarithmic mean	12.9 (13.3)	13.3 (14.4)	13.5 (14.7)	
Method (iii)				
Mean K_D	21.5 (27.0)	15.2 (16.3)	18.0 (21.5)	
s.e.mean	4.84 (7.75)	1.62 (2.86)	2.84 (4.98)	
Logarithmic mean	17.8 (18.7)	14.5 (14.3)	16.2 (17.4)	

B Mean values for all experiments with $Sd^2 < 2.5\%$ (5 in set 1 and 6 in set 2)

	Low			*High*	
Mean K	*s.e.mean*	*lmean*	*Mean K*	*s.e.mean*	*lmean*
(i) 12.4	1.07	11.9	12.6	1.22	12.0
(ii) 11.3	0.84	11.0	12.1	1.07	11.6
(iii) 16.5	2.21	15.2	13.1	1.39	12.3
For average of low and high:					
(i) 12.5	1.11	12.0			
(ii) 11.7	0.89	11.3			
(iii) 14.8	1.63	13.9			
For fit of two lines					
12.4	1.10	11.9			

effect of method in the analysis of variance of the values in Table 1. The slopes of the individual lines, however, indicate that slightly higher values are obtained with method (iii) than with methods (i) or (ii). This can be seen in Table 2A which also shows the greater variance of estimates obtained with method (iii), particularly with low agonist responses, probably because of the greater effect of errors on low dose-ratios (referred to in the Introduction). All three methods assume the validity of the Gaddum-Schild equation (1). This is all that is assumed by method (iii), which is why the estimate of K_D obtained by this method was used in the graphical check shown in Figure 4a and 4b. Methods (i) and (ii) also assume the validity of equation 2, from which equation 6 is derived, and should be expected to give similar estimates of K_D. The fit of both lines using method (i) seems to offer advantages because it obtains one value from all the data and the results of the experiments where $Sd^2 < 2.5\%$ shown in Table 2B give the best estimate of K_D.

The results in Table 2 indicate slightly higher activity for the compound than previous estimates. The value of K_D corresponding to the mean affinity constant obtained by Barlow *et al*. (1963) is 22.7 ± 0.9 (s.e.mean, $n = 4$) and the values corresponding to the mean estimate of log. affinity constant $\pm$ s.e.mean obtained by Abramson *et al*. (1969) are 26.4 nM, 24.8 nM and 28.2 nM, $n = 7$. These were obtained from experiments in which a range of antagonist concentrations was tested usually producing substantial dose-ratios (e.g. 5 to 100 or more) and the results used to calculate a single estimate for the tissue. Taking the average of estimates of log affinity constant for each concentration is the same as making a 'Schild-plot' (Arunlakshana & Schild, 1957) with 'the slope constrained to one' a procedure used, for instance, by Roberts *et al*. (1996). The condition for a least-squares fit of N results to the equation

$$\log\,(\mathrm{DR}-1) = \log[\mathrm{B}] + \mathrm{pA}_2$$

is that $N.pA_2 = S(\log(DR\text{-}1) - S(\log[B])$ (where S indicates the sum) so the fitted value of pA_2 is the average of $\log\{(DR\text{-}1)/[B]\}$.

The main aim of this work was to investigate the relation between Q and [B] and the experimental design, with alternate small and large responses, was chosen because with tissues, such as ileum, it is known that the response obtained with an agonist is affected by the previous response (Stephenson, 1956), particularly if this has produced a very small or a very large effect (Barlow, 1975). This plan also makes the experiments particularly suitable for measuring K_D using low concentrations of an antagonist and the results supplement the work of Lazareno & Birdsall (1993b, c), who have

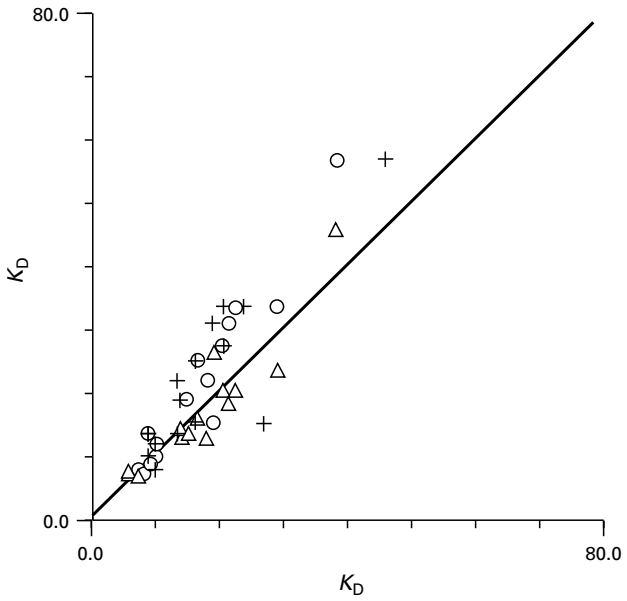

Figure 5 Estimates of K_D obtained by different methods: (○) indicate means of (19) values obtained by method (ii) for low and high agonist stimulation plotted against the fit to both lines by method (i): slope ($\pm$s.e.) = 1.05 ($\pm$0.09), constant = -0.69 ($\pm$1.67): (△) indicate means of values obtained by method (iii) for low and high agonist stimulation plotted against the fit to both lines by method (i): slope ($\pm$s.e.) = 1.47 ($\pm$0.11), constant = -3.45 ($\pm$1.97): (+) indicate means of values obtained by method (iii) for low and high agonist stimulation plotted against means of values obtained by method (ii) for low and high agonist stimulation: slope ($\pm$s.e.) = 1.23 ($\pm$0.15), constant = 0.23 ($\pm$2.72). Note that the points lie fairly evenly about the line of identity.

described methods for measuring K_D from inhibition curves obtained with antagonists on acetylcholine stimulated binding of [^{35}S]-GTPγS to CHO cells expressing human m1-m4 muscarinic receptors. Values of the exponent P (logistic slope factor) for the agonist with this system are not usually as high as with mechanical responses obtained with guinea-pig isolated ileum, however, which makes it easier to see with ileum, for instance, that there are conditions in which IC_{50} can be less than K_D.

The method by which K_D is calculated is less important than the experimental design but the results confirm that inhibition curves are suitable for measuring K_D with low concentrations of antagonist. This may be necessary because the compound is in short supply, has limited solubility or has other effects at high concentrations. To test for competitive behaviour an analysis of variance may be made (as in this work) on the results obtained with low and high agonist responses but departure from competitive behaviour must be difficult to detect when only a small range of antagonist concentrations is tested. If necessary, further experiments can be done in the presence of a known competitive antagonist (Ariens *et al*., 1964; Paton & Rang, 1965; Abramson *et al*., 1969).

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