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A comparison of effects measured with isotonic and isometric recording: II. Concentration-effect curves for physiological antagonists

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1 If one drug, B, antagonizes another, A, by producing the opposite physiological effect, the antagonist concentration-effect curves should be affected by the recording system, which limits the range of agonist responses.

2 With pieces of isolated guinea-pig ileum taken from adjacent parts of the same animal, one recorded isotonically, the other isometrically with the same load, the isotonic IC_{50} values for (−)isoprenaline opposing carbachol or histamine were lower than the isometric values ($P<0.01$) but there was a significant correlation between them ($P<0.01$): the isotonic curves were steeper ($P<0.01$) and there were wider shifts in IC_{50} before increasing the agonist reduced the maximum relaxation.

3 In similar experiments with pieces of rat uterus in oestrus from the same animal, the concentration-effect curves for carbachol opposed by increasing concentrations of (−)isoprenaline or (−)adrenaline had slightly lower EC_{50} values with isometric recording but there was a significant correlation ($P<0.01$) with isotonic values. The antagonist effect (ratio of the EC_{50} relative to that for the control) was higher with isotonic recording ($P<0.01$ for (−)isoprenaline, $P<0.025$ for (−)adrenaline) and all (27) curves were steeper than the corresponding isometric curve ($P<0.001$).

4 The influence of the method of recording on the results is expected from the narrower operational window and smaller upper limit to relaxation with isotonic recording.

5 A way of obtaining measurements of IC_{50} against a standard agonist effect is suggested in an Appendix.

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Abbreviations: *AS*, degree of agonist stimulation, $[A]/[A_{50}]$; E, agonist effect; M%, maximum effect expressed as % of controls: M_A, maximum effect of drug A; M_B, maximum effect of opposing drug B (equation 1); M/T ratio, ratio of isometric to isotonic values of IC_{50}; P, exponent in an empirical model for a concentration-effect curve (equations 3 and 4): P′, exponent obtained by fitting positive values obtained with equation 1; P_A, P_B, exponents for drugs A and B; RA, ratio of (agonist) EC_{50} in the presence of antagonist to control EC_{50}

Introduction

The shapes and positions of concentration-effect curves for antagonists depend on the agonist effects, which are being opposed. As has been shown in Part 1, these are affected by the method used for recording as well as by the concentration of agonist and this paper compares concentration-effect curves obtained with isotonic and isometric recording where one drug antagonizes another by producing the opposite physiological response. Common examples are relaxants or dilators, e.g. adrenaline, antagonizing constrictors, e.g. acetylcholine or histamine. Both types of drug are agonists but act at different receptors with different effector mechanisms, though these seem likely eventually to converge at the final step involving effects on the intracellular concentration of calcium ions. The situation has been variously described as 'physiological', 'independent' or 'functional' antagonism and although there has been much speculation about possible mechanisms these are not fully understood (Ariens *et al*, 1957; Van den Brink, 1973a, b; Mackay, 1981; Emmerson & Mackay, 1981; Hughes & Mackay, 1985; Goodall *et al*., 1986; Lew, 1995: Lew & Flanders, 1999).

The interaction of the two types of drug can be studied by obtaining concentration-effect curves for the agonist in the presence of increasing concentrations of antagonist: unlike the 'parallel shift' seen with competition, the antagonist reduces (possibly to zero) the maximum effect obtainable with the agonist. Alternatively, and possibly more commonly, an antagonist-effect curve is obtained by observing the reduction of the effect of a fixed concentration of agonist by increasing concentrations of antagonist. Although there is no established mechanism for the antagonism, two possibilities of what might be expected are illustrated in Figure 1. One supposes that the effect is the algebraic sum of two concentration-effect curves, so for two agonists, [A] and [B], acting independently and producing opposing effects, S_1 and S_2, the agonist effect,

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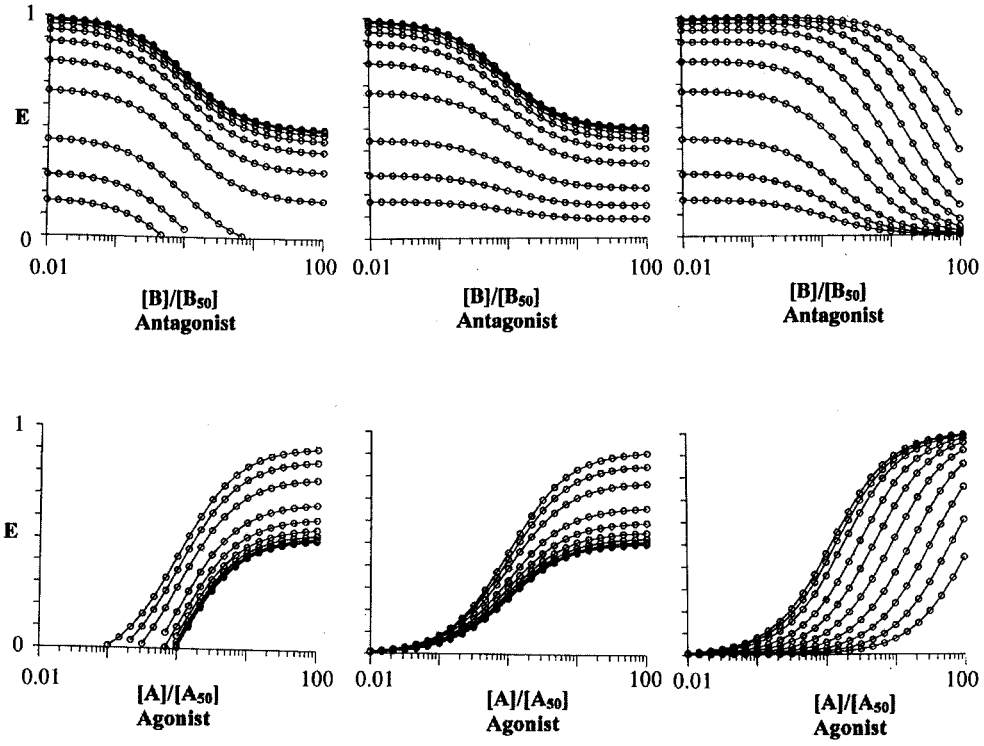

Figure 1 Agonist effect, E, for physiological antagonism (equation 1, left), functional antagonism (equation 2, centre) and competitive antagonism at a single receptor (right) and (upper) antagonist concentration, [B]/[B_{50}], for values of [A]/[A_{50}] from 0.2 to 128 and $M_A=1$, $M_B=0.5$, $P_A=P_B=1$. (Lower) agonist concentration, [A]/[A_{50}], for values of [B]/[B_{50}] from 0.2 to 128 and $M_A=1$, $M_B=0.5$, $P_A=P_B=1$.

$$E = S_1 - S_2 = \frac{M_A [A]^{PA}}{[A]^{PA} + [A_{50}]^{PA}} - \frac{M_B [B]^{PB}}{[B]^{PB} + [B_{50}]^{PB}} \quad (1)$$

where M_A and M_B are their respective maxima, [A_{50}] and [B_{50}] produce half these and P_A and P_B are the exponents for the two curves.

A second possibility, using a model proposed by Van der Graaf *et al.* (1996) assumes that negative effects are impossible and the second drug reduces the maximum obtainable with the first, e.g. M_A is multiplied by $(1-S_2)$, so:

$$E = M_A(1 - S_2)\frac{[A]^{PA}}{[A]^{PA} + [A_{50}]^{PA}} \quad (2)$$

With the first scheme E can be negative – the relaxation can be greater than the contraction – and the graphs of E against [B] are displaced towards higher concentrations as [A] is increased before further increases in [A] only reduce the maximum relaxation available without affecting IC_{50} (Figure 1 left). With the second scheme there is no shift; $IC_{50}=[B_{50}]$ (Figure 1 centre). If the antagonism involves two drugs competing for the same receptor, the picture is quite different (Figure 1 right).

The maximum effect of the antagonist is set by the threshold of the recording system so with isotonic recording, where the tissue cannot relax beyond its length extended by the load, the antagonist curve should be steeper and displaced towards lower concentrations (lower IC_{50}) than with isometric recording, where tension can still be recorded even though it falls below the initial value. As with the agonist curves (Part 1), however, the effect on IC_{50} may be more difficult to detect if the (antagonist) curve is steep.

When concentration-effect curves for [A] are obtained in the presence of increasing concentrations of [B] the situation should be similar if there is an upper limit above which any response is recorded as maximum. There will be a shift in the concentration of [A] producing a half-maximal response (EC_{50}) before there is no further shift and the maximum is reduced (Figure 1 lower). The shifts should be greater with isotonic recording, though this may be more difficult to see if the agonist concentration-effect curve is steep.

This paper describes the results of experiments with (−)isoprenaline antagonizing the effects of carbachol and histamine on guinea-pig ileum using isotonic and isometric recording with adjacent tissue samples taken from the same animal (as in Part 1). Comparisons between curves for antagonists with different methods of recording, however, should be made against the same agonist effect. For this reason it is convenient to 'normalize' the agonist concentration, replacing [A] by [A]/[A_{50}], which is referred to as the degree of agonist stimulation (*AS*; Barlow, 1995), and in each experiment curves were obtained for two levels of agonist effect, one less than ($AS<1$) and the other more than ($AS>1$) half the agonist maximum (obtained with $AS=1$). Experiments were also made with (−)isoprenaline and (−)adrenaline antagonizing the effects of carbachol on rat uterus in oestrus, which has a very steep agonist concentration-effect curve. These involved obtaining agonist concentration-effect curves in the presence of increasing concentrations of antagonist, using isotonic and isometric recording with

adjacent samples taken from the same animal. The aim in both types of experiment was to see if differences in the method of recording produced the expected differences in concentration-effect curves. A preliminary report of some of this work has appeared as an abstract (Barlow *et al*., 1999).

Methods

Details of the preparations have been given in Part 1 and all experiments began by obtaining a control concentration-effect curve for the agonist (Table 1, Part 1). In all experiments with guinea-pig ileum the Krebs' solution contained 0.1 mM hexamethonium and the control curve was used to calculate concentrations of agonist, which should produce roughly 30% ('low') and 70% ('high') of the maximum response. These were then automatically applied alternately (the contact time was 30 s with doses applied every 3 min) by computer-driven relays until the responses were regular. The Krebs' solution was replaced by solution containing the lowest concentration of antagonist: when the responses to low and high agonist were regular, the antagonist was increased, so a response curve for the antagonist was obtained (for at least six concentrations).

With the rat uterus the steepness of the agonist concentration-effect curves made it impossible to select concentrations, which gave consistent 'low' and 'high' responses so it was necessary to study the effects of increasing antagonist on agonist concentration-response curves. The agonist contact time was 45 s with doses applied every 3 min. When the control curve had been obtained, the de Jalon's solution was replaced by solution containing the lowest concentration of antagonist and the curve repeated. Carbachol chloride, hexamethonium bromide, histamine, (−)adrenaline and (−)isoprenaline were obtained from Sigma. All responses were recorded with a Maclab system.

Data analysis

In the work with guinea-pig ileum the effect of the antagonist can be expressed as the percentage inhibition of the control responses, rising from zero to a maximum, M%, or it can be taken as the actual size of the agonist effect, E, which falls from the control value to zero or a baseline. The data for a rising curve were fitted to the equation:

$$\%\ \text{inhibition} = M\% \frac{[B]^P}{[B]^P + [IC_{50}]^P} \quad (3)$$

and those for a falling curve to:

$$E = M \frac{[B]^P}{[B]^P + [IC_{50}]^P} + BL \quad (4)$$

to obtain estimates of M% (or M), [IC_{50}], P (which is negative for a falling curve) and BL using the program SPICE, (Bailey *et al*., 1998). With a falling curve the maximum fall (M) was expressed as a percentage (M%) of the control response. The method of analysis makes only small differences to the values of [IC_{50}] but the fit to a rising

Table 1

A Antagonist effect curves: numbers show the range of values [IC_{50}]($\times 10^{-8}$ M), P and M%

	Guinea-pig ileum					
	Isotonic			*Isometric*		
	IC_{50}	*P*	*M%*	IC_{50}	*P*	*M%*
Carbachol						
low *AS*	0.013 – 1.56	0.64 – 2.7	36 – 300	0.12 – 3.13	0.43 – 1.6	40 – 136
high *AS*	0.23 – 3.62	0.69 – 3.6	31 – 120	0.27 – 2.8	0.29 – 1.3	42 – 129
Histamine						
low *AS*	0.14 – 0.40	1.12 – 2.2	85 – 110	0.085 – 2.0	0.63 – 1.5	96 – 135
high *AS*	0.41 – 1.3	1.1 – 2.4	81 – 105	0.48 – 5.5	0.83 – 1.4	97 – 107

B Numbers show the ratio of the values of [IC_{50}], P and M% for high to low agonist stimulation and of [IC_{50}] for isometric and isotonic recording (M/T)

	Guinea-pig ileum							
	Isotonic			*Isometric*			*M/T ratio*	
	IC_{50}	*P*	*M%*	IC_{50}	*P*	*M%*	*low*	*high*
Carbachol								
C1	4.9	0.52	1.17	0.35	0.56	1.3	4.2	0.3
C2	20	1.7	0.85	0.97	0.77	1.05	5.8	0.3
C3	43	1.4	0.67	2.8	1.8	0.43	8.3	0.5
C4	3.2	1.8	0.26	0.79	0.94	1.04	3.4	0.8
C5	18	1.7	0.53	1.6	0.62	1.1	21	1.9
C6	1.3	0.97	1.7	2.1	0.49	1.6	0.3	0.4
C7	40	4.1	0.28	1.6	0.83	0.98	72	1.9
Histamine								
H1	3.2	1.7	0.85	2.7	0.73	1.1	5.0	4.4
H2	2.3	1.8	0.99	1.9	0.76	0.98	1.2	1.3
H3	2.4	0.82	1.02	1.9	0.90	1.04	1.9	1.5
H4	1.9	0.95	0.99	2.1	0.98	0.98	1.1	1.2
H5	4.5	1.5	0.94	10.6	2.1	0.74	0.6	1.4
H6	1.6	0.73	1.06	2.4	0.93	0.99	0.7	1.0

curve may overestimate M%. All results were eventually analysed in both ways. The fit to a falling curve should have the advantage that the conversion of the maximum effect to a percentage (M%) of the control response is the last step in the calculations (so errors in the controls should have less effect) but occasionally this fit would not converge and the values obtained with the fit to a rising curve were used.

Statistical tests A sign test was used to investigate differences between isotonic and isometric recording with tissues from the same animal. The significance of correlations was tested with both parametric (r, Student's t) and nonparametric (Spearman's rank coefficient) methods.

Results

An example of the concentration-effect curves obtained in an experiment with ileum is shown in Figure 2. As in the work of Van den Brink (1973a, b) there was great variation between experiments, and the range of values of the parameters describing the curves is shown in Table 1A. The results for any experiment were only excluded if the estimated value of IC_{50} was outside the range of concentration tested. In some experiments the antagonist appeared to produce more than 100% relaxation, possibly because the tissue became more sensitive to the agonist during the experiment, and with isometric recording possibly because the relaxation fell below the initial level but could still be recorded. In spite of the wide variation it is possible to reach some conclusions, particularly by making comparisons in individual experiments with tissue from the same animal.

The range of values of P (Table 1A) indicates that curves with isotonic recording are steeper than with isometric and comparing results obtained with tissue from the same animal this was true in 11 out of 14 instances with carbachol as agonist and in 10 out of 12 with histamine ($P<0.01$). The effects of histamine could be completely overcome by (−)isoprenaline in nearly all experiments, the lowest values of M% being 81% (isotonic) and 96% (isometric), whereas with carbachol there were experiments in which M% was as low as 31% (isotonic) and 40% (isometric); 0.1 mM hexamethonium was present with both agonists.

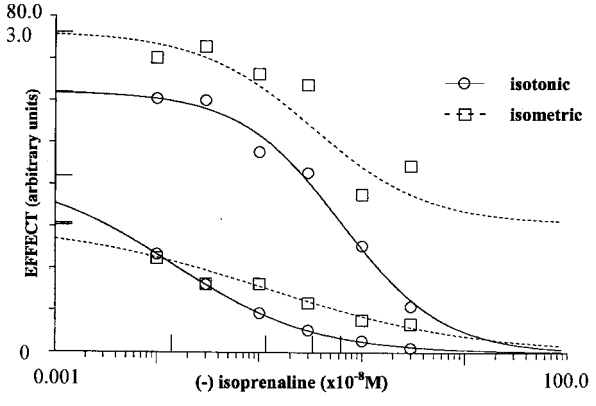

Figure 2 Concentration-effect curves for the effects of (−)isoprenaline on responses to carbachol with pieces of ileum from the same animal (C2), one recorded isotonically (low $AS=0.59$, high $AS=1.76$) and the other isometrically (low $AS=0.27$, high $AS=1.37$). Note the greater shift in IC_{50} with isotonic recording.

The effects of the method of recording on values of IC_{50} can be seen from the ratio (M/T in Table 1B) of the values for isometric recording compared with isotonic using tissue from the same animal: the values are higher (ratio > 1) in 19 out of 26 instances ($P<0.01$). For the results with carbachol, however, the IC_{50} ratio is higher in six out of seven comparisons with low agonist stimulation but only two out of seven with high stimulation (where the antagonist is less likely to produce negative responses recorded as zero). As with the results with agonists (Part 1), there is a significant correlation between the values obtained with isotonic and isometric recording (slope = 0.26; Spearman rank correlation coefficient = 0.55, $n=26$, $P<0.01$).

The effects of the degree of agonist stimulation on IC_{50}, P and M% can be seen from the ratio of the values for high and low stimulation shown in Table 1B. With isotonic recording all the ratios of IC_{50} are greater than one with higher agonist stimulation ($P=1$ in $2^{13}=0.0001$) but with isometric recording there were three instances with carbachol as agonist where this was not so.

Examples of the concentration-effect curves obtained in experiments with the rat uterus in oestrus are shown in Figure 3 and the results are summarized in Table 2. The curves obtained with isotonic recording are extremely steep, often with an exponent so high that it is necessary to choose units of concentration such that the numbers do not become too small or too large for the computer to handle. In Figure 3 it appears that the curve may be flatter at the top than at the bottom, so that a fit to equations 3 or 4 may not be strictly appropriate but this has little effect on values of EC_{50} and the equations give valid measures of the position and slopes of the curves so that comparisons can be made.

There are consistent differences in slope: all (27) curves are steeper with isotonic recording ($P<0.001$). Simply by comparing the method of recording on values of EC_{50} there does not seem to be a significant effect: in 10 out of 15 instances with (−)isoprenaline and in nine out of 12 instances with (−)adrenaline it is lower with isometric recording. The problem was investigated further, however, by comparing the mean values of the M/T ratio (Table 2) with one and the mean values of the log.ratio with zero. The mean log ratio is significantly different from 0 with the pooled data ($P<0.01$) and with the values for (−)adrenaline ($P<0.05$).

As in the experiments with guinea-pig ileum, there is a correlation between values of EC_{50} obtained by the two methods. For (−)isoprenaline the Spearman rank correlation coefficient was 0.79 ($P<0.01$): the slope of the correlation was 1.24 (s.e.mean 0.22, 15 points). For (−)adrenaline the coefficient was 0.80 ($P<0.01$) and the slope was 0.61 (s.e.mean 0.12, 12 points).

The effects of the antagonist can be expressed as a concentration-ratio (RA in Table 2) by comparing the value of EC_{50} in the presence of the antagonist with that of the controls. Antagonist concentration-effect curves are shown in Figure 4, in which this ratio is plotted against antagonist concentration. There are clear differences between (−)isoprenaline and (−)adrenaline but there are also differences between results with isotonic and isometric recording. The

Table 2 Effects of antagonist on agonist [EC_{50}]($\times 10^{-5}$ M), P and M%: RA is the ratio of [EC_{50}] to that of the controls and M/T the ratio for isometric to isotonic recording

	Rat uterus (carbachol $\times 10^{-5}$ M)								
	Isotonic					*Isometric*			
	EC_{50}	*P*	*M%*	*RA*	EC_{50}	*P*	*M%*	*RA*	*M/T*
					(−)isoprenaline				
RU1									
0.1 nM	1.17	7.01	93	1.2	0.98	3.65	84	0.8	0.8
0.3 nM	2.02	4.60	73	2.0	0.83	2.24	74	0.7	0.4
RU2									
0.1 nM	1.16	12.6	98	3.2	0.65	2.66	96	1.5	0.6
0.3 nM	1.15	5.9	94	3.2	1.14	5.42	87	2.6	1.0
1.0 nM	1.91	6.4	92	5.3	1.34	2.58	84	3.0	0.7
3.0 nM	2.21	5.3	95	6.2	1.60	2.02	86	3.6	0.7
RU3									
1.0 nM	4.51	5.0	72	4.7	6.98	1.25	79	8.3	1.5
RU4									
0.1 nM	0.40	8.6	98	1.5	0.89	1.48	113	2.0	1.7
1.0 nM	0.70	10	96	2.6	0.75	1.54	121	1.7	1.1
3.0 nM	1.97	6.4	94	7.4	1.15	1.88	117	2.6	0.6
10 nM	3.55	8.0	89	13	2.45	2.57	89	5.5	0.7
RU5									
0.1 nM	1.2	10	100	3.1	1.11	3.4	98	2.1	0.9
0.3 nM	1.25	10	99	3.3	1.20	3.2	98	2.2	0.9
3.0 nM	2.09	10	96	5.4	1.54	3.0	89	2.9	0.7
10 nM	2.00	10	89	5.2	204	3.3	78	3.9	1.0
Mean EC_{50} ratio (M/T) = 0.887, s.e.mean = 0.089: mean log ratio = −0.080, s.e.mean = 0.041.									
					(−) adrenaline				
RU6									
10 nM	1.26	10	92	1.9	1.06	4.4	91	1.6	0.8
0.1 μM	1.23	10	90	1.8	0.89	2.2	101	1.4	0.7
1.0 μM	5.95	2.9	46	8.9	3.19	2.4	44	4.9	0.5
1 mM	6.55	6.6	43	9.8	5.42	4.3	25	8.4	0.8
RU7									
1.0 nM	1.56	6.0	104	4.0	0.78	5.3	58	2.3	0.6
1.0 μM	3.8	3.4	53	9.7	2.73	1.8	32	8.0	0.7
100 μM	3.72	3.5	73	9.5	2.68	2.7	27	7.9	0.7
RU8									
1 nM	2.69	3.9	91	2.7	1.30	3.0	83	1.7	0.5
RU9									
1 nM	1.32	7.7	92	2.0	2.45	2.2	73	0.9	1.9
0.1 μM	2.10	5.4	85	3.2	2.31	3.3	53	5.0	1.1
RU10									
1 nM	1.98	4.4	96	1.9	2.06	1.8	84	2.3	1.0
0.1 μM	2.90	4.0	84	2.8	2.39	2.1	69	2.6	0.8
Mean EC_{50} ratio (M/T) = 0.842, s.e.mean = 0.109: mean log ratio = −0.105, s.e.mean = 0.046. Overall mean = 0.867, s.e.mean = 0.068; mean log ratio = −0.091, s.e.mean = 0.030.									

ratio was higher with isotonic recording in 13 out of 15 comparisons with (−)isoprenaline ($P<0.01$) and with 10 out of the 12 with (−)adrenaline ($P<0.025$).

Discussion

The great variation in the effects of the antagonists is similar to that found in the experiments of Van den Brink (1973a, b) but as with the experiments in Part 1 the experimental plan makes it possible to detect differences between results obtained with the two methods of recording even though there is a wide range in the sensitivity of tissues from different animals.

In both types of experiment concentration-effect curves are steeper with isotonic recording. In the experiments with ileum the values of IC_{50} are lower with isometric recording and increased agonist stimulation produces a bigger increase with isotonic than with isometric recording (M/T ratio). These effects are expected because with isotonic recording there can be less relaxation than with isometric.

These conclusions have been reached by comparing values obtained with the antagonist tested against 'low' and 'high' agonist effects on the same tissue without taking account of

the actual values of AS in each experiment. Because the concentration-effect curve for the agonist is steeper with isotonic recording, the increase needed to go from 'low' to 'high' effects is smaller. Accordingly, the larger changes in AS with isometric recording reinforce the flatness of the correlation between isometric and isotonic values of IC_{50} obtained with ileum (slope = 0.26: see Results). Isotonic recording definitely produces a greater shift. A possible way of making comparisons between drugs or tissues by expressing values of IC_{50} for a standard agonist effect, such as $AS = 1$ (where the agonist alone should produce a half-maximal response) is suggested in the Appendix.

In the experiments with rat uterus, the lower values of EC_{50} obtained with isometric recording were not expected but are probably explained by the extreme steepness of the curve – the response is almost all-or-none – and the apparent lack of symmetry between the lower and upper parts. With isotonic recording there appears to be a threshold below which there is no response (Figure 5) and above which there is likely to be a synchronized contraction of the tissue. The

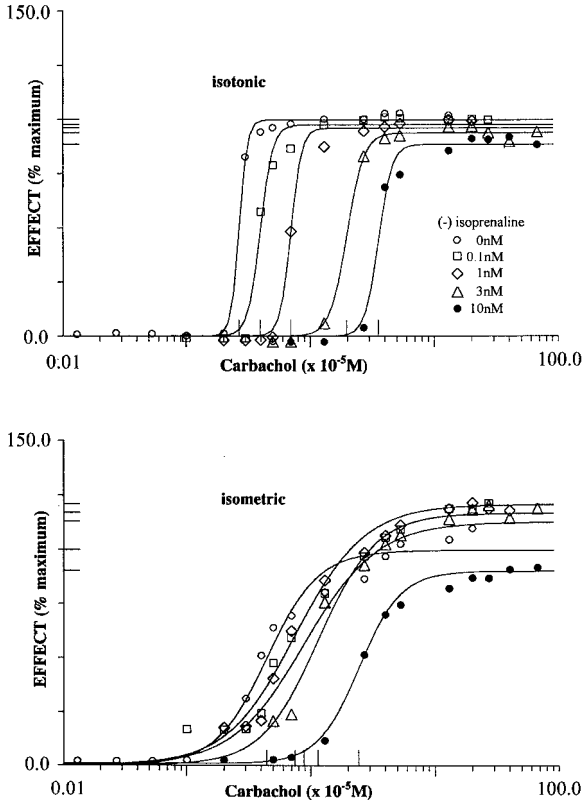

Figure 3 Concentration-effect curves for carbachol on rat uterus (RU4) in the presence of increasing concentrations of (−) isoprenaline with isotonic and isometric recording using tissues from the same animal. Note the flatter curves with isometric recording.

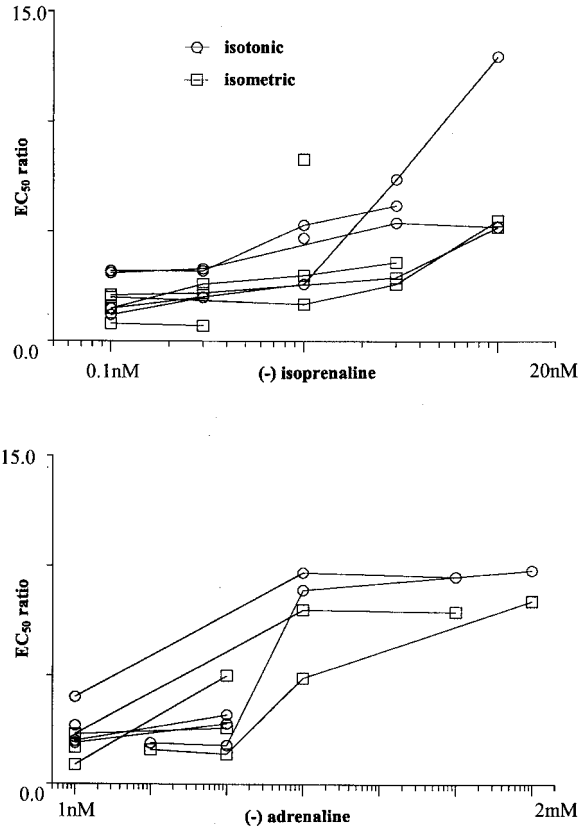

Figure 4 Antagonist concentration-effect curves for (−)isoprenaline and (−)adrenaline where the effect is measured as the EC_{50} ratio for carbachol relative to the control curve: there are bigger effects with isotonic recording.

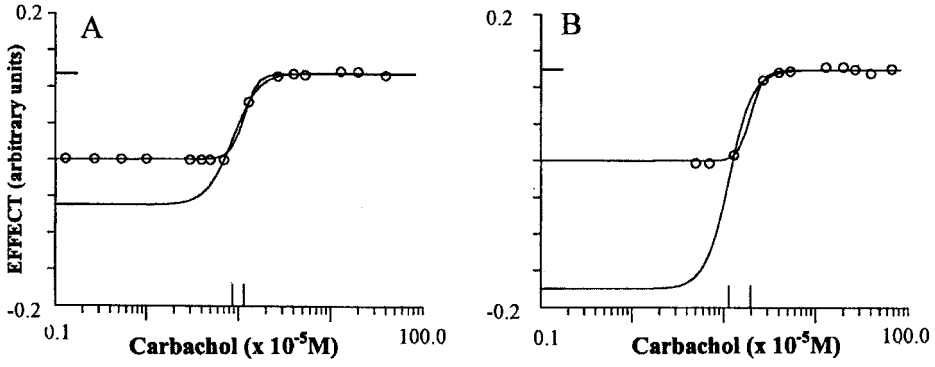

Figure 5 Concentration-effect curves for carbachol on rat uterus recorded isotonically showing the effects of omitting values close to zero, which indicate a substantial threshold. Results are for RU2 with 1 nM (−)isoprenaline (A) and RU4 with 3 nM (B). With all points included the curve is steeper ($P = 5.9$ compared with 3.1 and 6.4 compared with 3.4) and EC_{50} is higher (11.5 μM compared 8.6 and 19.7 μM compared with 11.4).

system accordingly operates within a window, which excludes the lower part of the signal curve and so shifts, the EC_{50} towards higher concentrations (see Part 1).

The significant correlation between the EC_{50} values obtained by the two methods has a slope close to one for (−)isoprenaline but less than one for (−)adrenaline, which has more effects on the maximum response to carbachol, even though higher concentrations are needed. With a competitive antagonist the EC_{50} ratio, RA, is a valid measure ('dose-ratio') of its effect at the concentration used: with the compounds used in this work it is not, because they reduce the maximum response obtainable with the agonist. They can serve, nevertheless, as a measure of the shift and show that the pattern is quite different from competition. With a ratio of two produced by 0.3 nM, a competitive antagonist should produce a ratio of 11 with 3 nM and 101 with 30 nM.

As in the other type of experiments, there was great variation between animals and the ratio (RA) is bigger with isotonic recording (see above and Figure 4). This is to be expected because with a higher threshold for isotonic recording, negative effects recorded as zero occur at lower concentrations (see Figure 1, lower left). This should be associated with a smaller reduction in M%, which is certainly true for the experiments with (−) adrenaline.

In conclusion, the work not only shows the influence of the method of recording on concentration-effect curves but also the results to be expected with this type of antagonism: it is important because many drugs affecting airways and blood flow act in this way. The results suggest a possible mechanism that the two types of drugs produce opposing effects on a common effector, such as the internal calcium ion concentration (see Part 1), which operates between limits.

References

ARIENS, E.J., VAN ROSSUM, J.M. & SIMONIS, A.M. (1957). Affinity, intrinsic activity and drug interactions. *Pharmacol. Rev.,* **9,** 218–236.

BAILEY, S.R., BARLOW, R.B., ELLIOTT, J. & GRAY, G.A. (1998). SPICE – a simple program identifying curvaceous elements. *Br. J. Pharmacol.,* **124,** 142P.

BARLOW, R.B. (1995). Use of an antagonist for estimating the degree of agonist stimulation during physiological release. *Trends Pharmacol. Sci.,* **16,** 262–264.

BARLOW, R.B., BOND, S.M., GRANT, C., MCQUEEN, D.S., SMART, J. & YAQOOB, Z. (1999). Operational windows make functional antagonism appear independent. *Br. J. Pharmacol.,* **128,** 302P.

EDINBURGH STAFF. (1970). *Pharmacological Experiments on Isolated Preparations,* 2nd edn. p 2, 14–17, 58–60, 94–95. Edinburgh: Churchill-Livingstone.

EMMERSON, J. & MACKAY, D. (1981). A test of the null equation for functional antagonism. *Br. J. Pharmacol.,* **73,** 135–141.

GOODALL, J., HUGHES, I.E. & MACKAY, D. (1986). Quantification of the actions of agonists that simultaneously act on a particular type of receptor and have separate functional interactant properties. *Br. J. Pharmacol.,* **88,** 639–644.

HUGHES, I.E. & MACKAY, D. (1985). Quantification of the actions of antagonists exhibiting both competitive antagonism and functional interaction. *Br. J. Pharmacol.,* **85,** 271–275.

LEW, M.J. (1995). Extended concentration- response curves used to reflect full agonist efficacies and receptor occupancy-response coupling ranges. *Br. J. Pharmacol.,* **115,** 745–752.

LEW, M.J. & FLANDERS, S. (1999). Mechanisms of melatonin-induced vasoconstriction in the rat tail artery; a paradigm of weak vasoconstriction. *Br. J. Pharmacol.,* **126,** 1408–1418.

MACKAY, D. (1981). An analysis of functional antagonism and synergism. *Br. J. Pharmacol.,* **73,** 127–134.

VAN DEN BRINK, F.G. (1973a). The model of functional interaction I Development and first check of a new model of functional synergism and antagonism. *Eur. J. Pharmacol.,* **22,** 270–278.

VAN DEN BRINK, F.G. (1973b). The model of functional interaction II Experimental verification of a new model: the antagonism of ß-adrenoceptor stimulants and other agonists. *Eur. J. Pharmacol.,* **22,** 279–286.

VAN DER GRAAF, P.H., SHANKLEY, N.P. & BLACK, J.W. (1996). Analysis of the effects of a_1 adrenoceptor antagonists on noradrenaline-mediated contraction of rat small mesenteric artery. *Br. J. Pharmacol.,* **118,** 1308–1316.

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Appendix

A method for standardizing the effects of physiological antagonists.

In experiments with values of IC_{50}, M and P obtained for $AS<1$ and $AS>1$, the values for $AS=1$ must lie between the two and as a first approximation might be calculated by simple proportion. Another possibility is that there is a linear relation with log.AS, so values could be calculated as in a three-point bioassay which assumes a linear relation between response and log.dose (e.g. Edinburgh Staff, 1970). There are reasons (discussed below) for believing that this is more appropriate but when the range of values of AS is small the differences between the two methods are also small. With the results shown in Figure 2 the two pairs of curves become the two lines shown in Figure 6: for the isotonic results with 'low' agonist $AS=0.59$, $IC_{50}=0.014$ ($\times 10^{-8}$ M), M% = 165, $P'=-0.64$; with 'high' agonist AS = 1.76, $IC_{50}=0.62$, M% = 110, $P'=-0.88$, so the interpolated values for $AS=1$ (with those assuming a linear relation with log.AS in parentheses) are $IC_{50}=0.23$ (0.31), M% = 146 (138), $P'=-0.73$ (−0.76). With the isometric results 'low' agonist $AS=0.27$, $IC_{50}=0.12$, M% = 136, $P'=-0.43$; with 'high' agonist AS = 1.37, $IC_{50}=0.33$, M% = 58, $P'=-0.79$, and the interpolated values for AS = 1 are $IC_{50}=0.26$ (0.29), M% = 84(73), $P'=-0.67$ (−0.72).

Values of IC_{50}, P′ and M% for $AS=1$ obtained from the results in Table 1 assuming a linear relation with log.AS are shown in Table 3, which includes mean estimates of log.IC_{50} calculated assuming a linear relation with AS for comparison. In all experiments with histamine and isotonic recording the curve is steeper and IC_{50} is lower ($P=0.016$) but there is little effect on M%. With carbachol, which is a stronger agonist, there is a reduction in M% with isometric recording and the effects on IC_{50} and P′ are less clear. The same conclusions are obtained by examining the (slightly different) values obtained assuming a linear relation with AS and reinforce the findings already presented.

Values of pIC_{50} for $AS=1$ are useful because they indicate the concentrations at which antagonistic effects may be expected (comparable with pD for a single agonist). The standard errors of mean values of pIC_{50} are around 0.3 compared with less than 0.1 for log.antagonist constants in competition experiments. If they

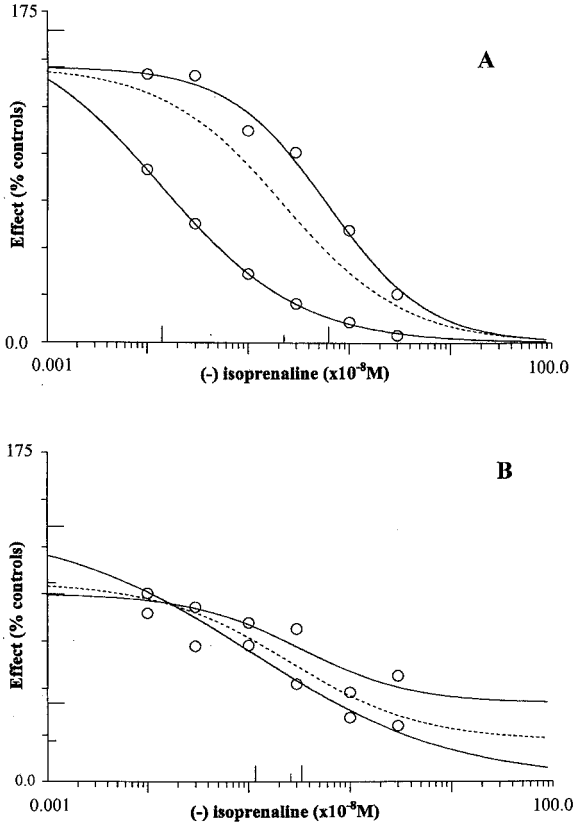

Figure 6 Concentration-effect curves (see Figure 2) for (−)isoprenaline on responses to carbachol (as percentage of controls) with pieces of ileum from the same animal (C3) showing the interpolated curve for $AS=1$ (broken line): (A), recorded isotonically (low $AS=0.59$, high $AS=1.76$) and (B), recorded isometrically (low $AS=0.27$, high $AS=1.37$).

are combined with the effects on M% the results for an individual tissue can be represented as a single point, which makes it easy to compare groups visually, as in Figure 7.

The estimation of values for $AS=1$ by interpolation is supported both by calculations with theoretical data and by the experimental results. With the model of antagonism represented by equation 1 the shifts in antagonist concentration curves will be independent of the steepness of the agonist curve P_A when $E=0$ (above which the antagonist should produce a negative response) or $E=0.5$ ($AS=1$). The relations between AS and [IC_{50}] can be obtained by generating theoretical antagonist concentration-effect curves from equation 1, assigning values to M_A, M_B, P_A and P_B, calculating E for increasing concentrations of antagonist and a particular value of AS, and fitting positive values to equations 3 or 4 to obtain estimates of M, [IC_{50}], P and BL. The relation between [IC_{50}] and AS (on a logarithmic scale, Figure 8A) appears to be sigmoid and roughly linear in the range $AS=0.5-2.0$. The results with guinea-pig ileum provide some support for this idea. In each experiment the actual values of AS for 'low' and 'high' agonist stimulation are known (from the concentration of agonist and the control responses). With isotonic recording there is a significant positive correlation ($P<0.05$) between IC_{50} and AS (Table 4), even

Table 4 Correlations between IC_{50} and agonist stimulation (AS) in experiments with guinea-pig ileum: T = isotonic recording: M = isometric

	Slope of correlation (s.e.mean)	*Number of points*	*Correlation coefficients* r (students *t*)	*Spearman*
		(−)isoprenaline and carbachol		
T	0.52 (0.27)	14	0.49 (1.96)	0.53*
M	−0.25 (0.34)	14	−0.20 (0.72)	−0.11
		(−)isoprenaline and histamine		
T	0.21 (0.11)	12	0.53 (1.99)	0.65*
M	0.49 (0.62)	12	0.24 (0.79)	0.42

*$P<0.05$.

Table 3 Guinea-pig ileum: interpolated values (concentrations $\times 10^{-8}$ M) for $AS=1$ from results in Table 1 assuming a linear relation with log.AS or with AS (italics)

	Isotonic			*Isometric*		
	IC_{50}	*P*	*M%*	IC_{50}	*P*	*M%*
Carbachol						
C1	1.33	−1.20	106	1.37	−0.45	124
C2	1.68	−1.00	95	1.04	−1.52	41
C3	0.31	−0.76	138	0.29	−0.72	73
C4	0.20	−1.39	80	0.30	−0.94	69
C5	0.011	−0.76	150	0.29	−1.20	71
C6	1.75	−2.70	44	0.36	−0.60	61
C7	0.37	−1.50	249	2.62	−0.93	83
mean	0.81	−1.33	123	0.90	−0.91	74.6
s.e.mean	0.28	0.25	25	0.33	0.14	9.6
p[IC_{50}]	8.43 (0.29) *8.52 (0.28)*			8.21 (0.15) *8.21 (0.15)*		
Histamine						
H1	0.74	−1.81	90	4.70	−1.23	104
H2	0.45	−1.53	84	0.78	−1.22	97
H3	0.53	−2.03	104	0.89	−1.43	99
H4	0.25	−1.19	103	0.40	−0.83	108
H5	0.26	−1.51	108	0.68	−1.11	110
H6	0.25	−2.24	90	0.45	−1.15	99
mean	0.41	−1.72	96.5	1.32	−1.16	103
s.e.mean	0.08	0.16	4.0	0.68	0.08	2.2
p[IC_{50}]	8.42 (0.08) *8.45 (0.08)*			8.07 (0.16) *8.13 (0.16)*		

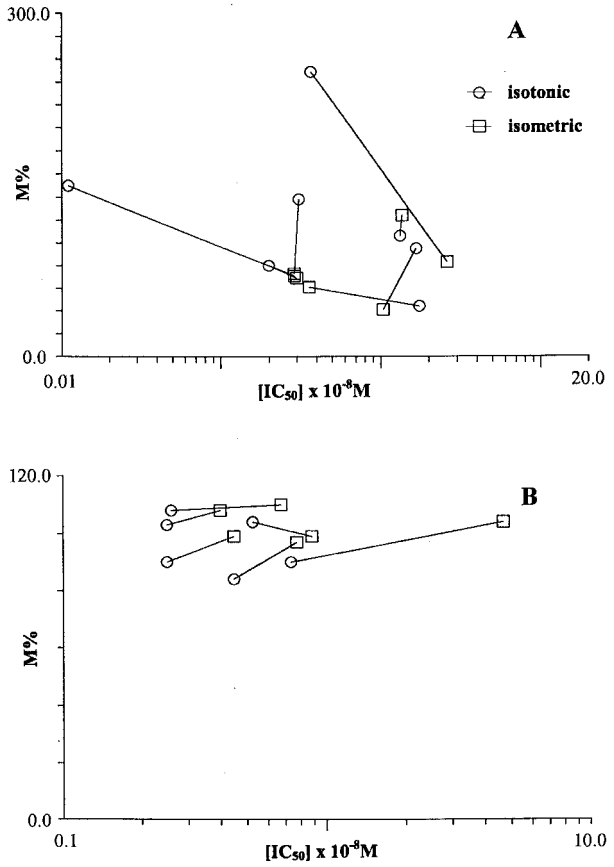

Figure 7 Experiments on guinea-pig ileum with carbachol (A) and histamine (B) as agonists: the interpolated value of M% for $AS=1$ is plotted against the corresponding value of IC_{50} and the lines join results obtained with isotonic and isometric recording with tissue from the same animal. Note the wide range of effects with carbachol on M% and IC_{50}, which is lower with isotonic recording in five out of seven instances. With histamine the results are more consistent (note the scales on both axes): there is little effect on M% (histamine is a weaker agonist than carbachol) and IC_{50} is lower with isotonic recording in all six comparisons ($P=0.016$).

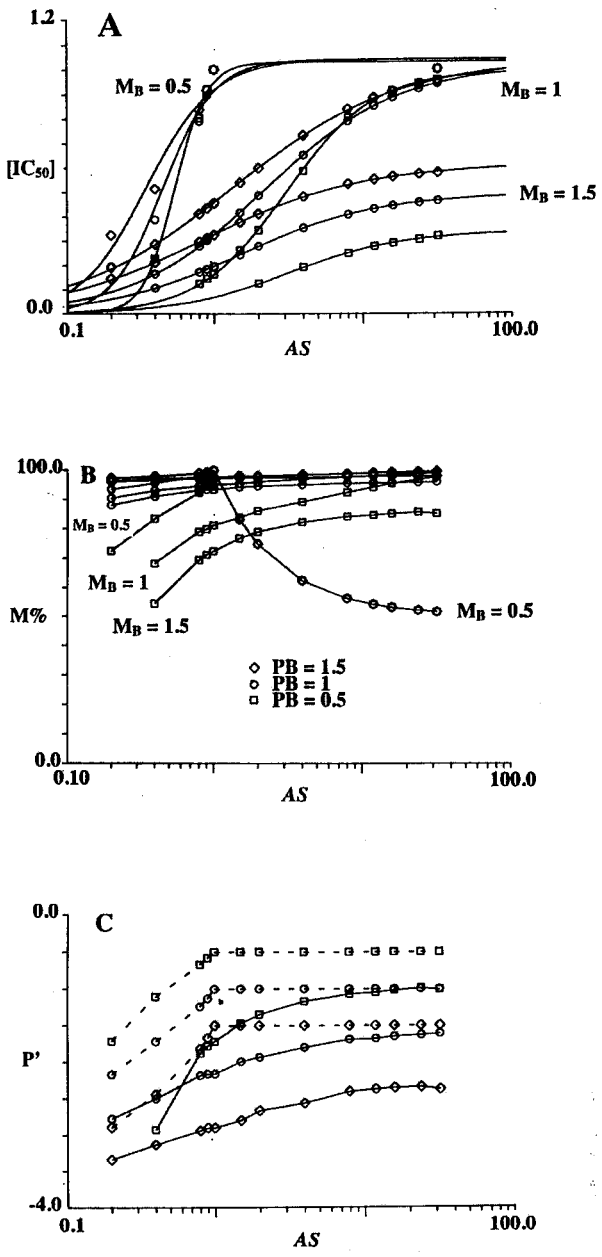

Figure 8 The effects of agonist stimulation, *AS*, on antagonist concentration-effect curves: positive values of E generated from equation 1 were fitted to equations 3 or 4, with $M_B/M_A=0.5$, 1 and 1.5 and $P_B=0.5$, 1 and 1.5. (A) Effects on [IC_{50}]: with a weak antagonist ($M_B/M_A=0.5$) [IC_{50}] is constant above $AS=1$ but with stronger antagonists there are shifts across a wide range. (B) Effects on M%: with a weak antagonist ($M_B/M_A=0.5$) M% falls above $AS=1$. (C) Effects on P′ for $M_B=0.5$ (broken line) and $M_B=1.5$: curves are steeper where there are shifts in [IC_{50}].

though this has involved pooling results from different animals (for which the ratios of M_B/M_A are likely to differ).

The relations between *AS* and M% and between *AS* and P are shown similarly in Figure 8B,C. With a weak antagonist (e.g. $M_B/M_A=0.5$) values of $AS>1$, which have no effect on IC_{50}, produce a progressive decrease in M% and the exponent, P, for the concentration-effect curve becomes that of the antagonist, independent of that of the agonist. When the ratio M_B/M_A is higher (e.g. one or more) and the agonist curve has $P=1$ or 1.5, M% is roughly 100%, but the concentration-effect is steeper than that of the antagonist (because of the limit to the effect which can be detected which is producing shifts in IC_{50}). With a flat agonist curve ($P=0.5$) low values of *AS* give values of M% less than 100% but this is misleading: if the antagonist effect is measured as an upward curve (as inhibition or relaxation with *P* positive), values of $M\%>100$ are obtained.

Just as in experiments with competitive antagonists it is usually impossible to obtain a concentration (dose)- ratio of exactly two in the measurement of pA_2, it is impossible to obtain results (M%, [IC_{50}], P) for the precise standard value, $AS=1$ with physiological antagonists. Although the calculation of values by interpolation may be an over-simplification, the experimental design involves obtaining an agonist control curve and testing the antagonist against two levels of agonist stimulation reasonably spaced below and above $AS=1$. This by itself goes some way towards standardizing conditions in which different antagonists may be compared.