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ON THE SIMULTANEOUS ACTION OF TWO COMPETITIVE ANTAGONISTS

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1 A hypothesis is outlined predicting the conditions in which the addition of a second competitive antagonist will increase rather than reduce the response to an agonist.

2 Experiments were performed with the guinea-pig ileum as the test tissue, hexyltrimethyl ammonium as the agonist, benzilyltropine methiodide as the 'slow' antagonist and pentyltriethyl ammonium as the 'fast' antagonist.

3 The results are consistent with the hypothesis, if the affinity constant for hexyltrimethyl ammonium is between 2.7 and 3.7×10^4 M^{-1}, if the dissociation time constant for the slow antagonist is greater than 10 min and if that for the fast antagonist is less than 10 seconds.

Introduction

Experiments have been described which show that in appropriate circumstances the addition of a 'second' competitive antagonist to a system containing a 'first' competitive antagonist, may increase, rather than further reduce, the response to an agonist (Stephenson & Ginsborg, 1969). The conditions in which such an effect is to be expected are:

(1) the first antagonist dissociates from the receptors slowly in relation to the exposure time to the agonist. The second antagonist and the agonist equilibrate with the receptors rapidly;

(2) each of the three drugs concerned is present in a concentration which would be sufficient for an appreciable proportion of the receptors to be occupied by that drug, if it were the only one present.

The experiments were based on the following hypothesis. In the presence of the slowly dissociating antagonist the receptors it occupies are, for the most part, unavailable to the agonist. When the fast antagonist is introduced, and allowed to equilibrate, fewer receptors are occupied by the slow antagonist. Although there are fewer receptors free when it is first added, the agonist will occupy more receptors than before as it equilibrates with the rapidly acting antagonist. The results previously reported were in qualitative agreement with this idea, and the present purpose is to explore the hypothesis more quantitatively. However, a serious difficulty is that there is no accurate information about several of the parameters required to calculate the degree of potentiation to be expected. What has been done therefore, is to explore by calculation, the ranges for these parameters within which the hypothesis can account for experimental observations.

Notation

The agonist of low efficacy was hexyltrimethyl ammonium bromide (hexyl TMA), and that of high efficacy was pentyltrimethyl ammonium bromide (pentyl TMA). The slow antagonist was benzilyltropine methiodide bromide and the fast was pentyltriethyl ammonium bromide (pentyl TEA).

The following notation will be used

A	agonist of low efficacy, hexyl TMA
C	agonist of high efficacy, pentyl TMA
S	slowly dissociating antagonist, benzilyltropine methiodide
F	fast antagonist, pentyl TEA
K_A	affinity constant for A (M^{-1})
[A]	molar concentration A
τ_A	dissociation time constant for drug A
c_A	($= K_A \cdot [A]$) normalized concentration corresponding to [A]
$p_A(S)$	proportion of receptors occupied ('occupancy') by [A] in presence of [S]
$p_A(S, F)$	occupancy by [A] in presence of [S] + [F]
$[C]_1$	concentration of C that matches [A] in the presence of [S]
$[C]_1'$	concentration of C that matches [A]′ in the presence of [S]
$[C]_2$	concentration of C that matches [A] in the presence of [S] + [F]
$[C]_2'$	concentration of C that matches [A]′ in the presence of [S] + [F]

p_1 occupancy corresponding to $[C]_1$
p'_1 occupancy corresponding to $[C]'_1$
p_2 occupancy corresponding to $[C]_2$
p'_2 occupancy corresponding to $[C]'_2$
R ratio of occupancy by concentration of C which matches [A] in presence of [S] + [F] to that which matches [A] in presence of [S] alone, i.e. $R = p_2/p_1$
R' p'_2/p'_1

Theory

Suppose that the interaction of drugs and receptors is governed entirely by simple mass action. If a tissue is simultaneously exposed to a number of drugs each of which can combine reversibly with one kind of receptor the proportion of receptors occupied by each drug (i.e. its occupancy, p', p'' . . .) at time t, is given by the solution to an equation of the kind:

$$\tau_A \frac{dp'}{dt} = c_A(1 - p' - p'' - \ldots) - p' \qquad (1)$$

where τ_A is the time constant of dissociation of the drug A with occupancy p' at time t and c_A is the 'normalized' concentration (i.e. $c_A = K_A[A]$ where K_A is the affinity constant, and where [A] is the molar concentration; see e.g. Paton, 1961; Rang, 1966; Colquhoun, 1968). Thus the agonist occupancy may be calculated, at the end of a specified exposure period with previous equilibration to one antagonist, by solving a pair of simultaneous equations and, with previous equilibration to two antagonists, from a set of three such equations. The nature of the solutions is conveniently illustrated graphically in the form of log concentration–occupancy curves as in Figure 1. In Fig. 1a, the solid line shows the computed relationship between the occupancy $p_A(S)$ and the normalized concentration c_A, in the presence of a particular concentration of a slowly-dissociating antagonist S with an assumed dissociation time constant τ_S, equal to 10 times the exposure time to the agonist. The dashed line shows new values for occupancy $p_A(S, F)$ after the addition and equilibration of a second, fast-dissociating, antagonist F, which has a dissociation time constant τ_F equal to 0.01 times the exposure time. The agonist has also been assumed to have a dissociation time constant τ_A of 0.01 times the exposure time. It can be seen that when c_A is greater than about 2, corresponding to a value of $p_A(S)$ of about 0.08, $p_A(S, F)$ exceeds $p_A(S)$; in other words a ('paradoxical') potentiation of the agonist occupancy would occur, with these assumptions. This contrasts with the situation usually considered (but see Rang, 1966) where

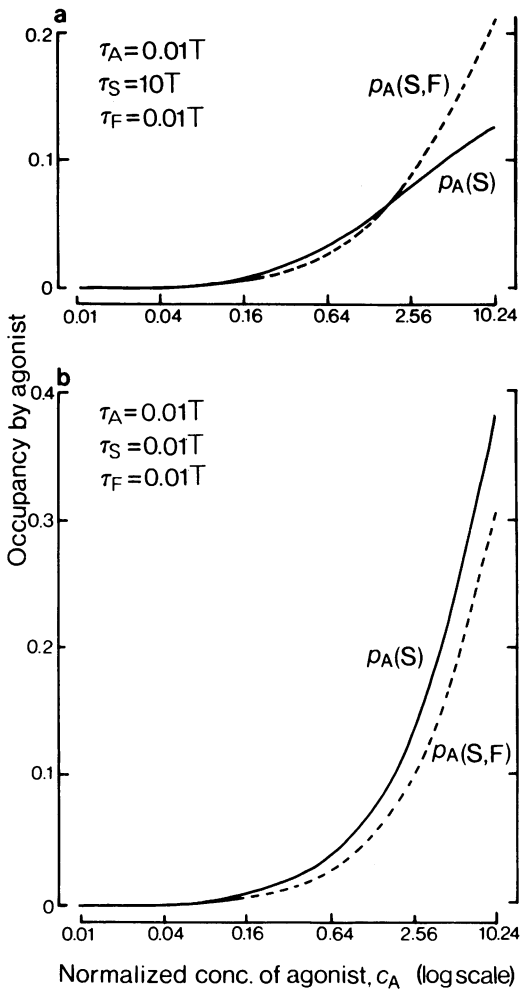

Fig. 1 Theoretical relationship between occupancy by agonist at end of exposure time T, and log-normalized concentration of agonist in the presence of one (S) antagonist or two (S and F) antagonists (dashed line) in fixed concentrations. If each were present alone S would occupy 0.937 and F, 0.871. For details relating to the time constants τ_A, τ_S, and τ_F see text. Curves were computed and drawn as described on p. 290.

equilibration between drugs and receptors is regarded as instantaneous. In such a case, of course, the addition of the second antagonist causes a reduction in occupancy by the agonist no matter what its concentration. Figure 1b illustrates this conventional state of affairs for the same concentrations of S and F but where τ_A, τ_S and τ_F are all negligibly small compared with the exposure time to the agonist.

If the concentrations of S and F are sufficient

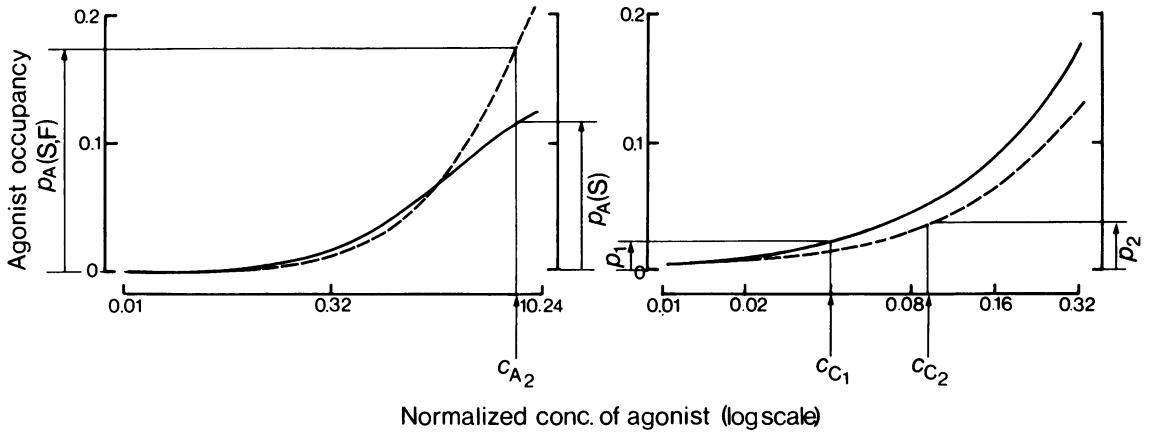

Fig. 2 Comparison of occupancy *v.* log-normalized concentration curve for an agonist of low efficacy (A) with one for an agonist of high efficacy (C), in the presence of one (S) antagonist (solid line) or two (S and F) antagonists (dashed line). The dissociation time constants are: $\tau_A = \tau_F = 0.01T$ and $\tau_S = 10T$; the antagonist occupancies are as in Figure 1. If the response to [A] is the same as the response to $[C]_1$ in the presence of S only and to $[C]_2$ after equilibration with F also, then $p_A(S, F)/p_A(S) = p_2/p_1$. See text and also caption to Figure 1.

(see Appendix) a paradoxical increase in response is to be expected when the initial occupancy by the agonist is larger than a critical value. The effect, however, would not be observed if the agonist produced the maximum response at an occupancy smaller than this critical value. Thus in the example illustrated in Fig. 1, if the efficacy (see Stephenson, 1956) of the agonist is such that a maximum response is obtained with an occupancy of less than 0.08, the only effect of the addition of the second antagonist will be to depress submaximal responses. Where an agonist produces responses at *sufficiently* small occupancies, the effect of the antagonists will be independent of their time constants (see below and also Rang, 1966). Thus the responses to an agonist of sufficiently high efficacy can be used as a measure by which to judge the potentiation of the agonist A.

What is required is to find the concentrations of the agonist of high efficacy C which match the responses to a known concentration of the agonist A of low efficacy; values for the matching concentrations are required in the presence of the slow antagonist alone and of both antagonists together. In the presence of the slow antagonist alone, let the known concentration [A] of the agonist A be matched by the concentration $[C]_1$ of the agonist C of high efficacy; suppose also that [A] occupies a proportion $p_A(S)$ of the receptors and that $[C]_1$ occupies p_1. In the presence of both antagonists let [A] occupy p_A and be matched by the concentration $[C]_2$ of C, which occupies p_2 (see Figure 2). Then (according to Stephenson, 1956), $p_A(S, F)/p_A(S)$ is equal to p_2/p_1. If the efficacy of C is sufficiently high, the second ratio R, say, is almost entirely independent of the dissociation time constant of C (τ_C) and of those of the antagonists (see below and also Rang, 1966), and R may be calculated from the concentrations $[C]_1$ and $[C]_2$ and the affinity constants alone. The value of R thus found constitutes the experimental estimate of $p_A(S, F)/p_A(S)$. This estimate may then be compared with values predicted for various assumed values for the dissociation time constants.

In practice it was found useful to determine two pairs of concentrations of C to match two different concentrations of A, [A] and [A′]. Apart from yielding two values, R and R′ to be compared with prediction, it allowed the estimation of the ratio of the occupancies by [A] and [A]′ in the presence of S alone. This ratio depends on τ_A, τ_S, K_A and K_S and may therefore be expected to provide some information about one or more of these parameters.

A greater physical insight into 'paradoxical' potentiation may perhaps be gained from the Appendix, section A, which discusses the limiting case where τ_A, τ_C and τ_F are zero and τ_S is infinite.

Methods

Guinea-pig ileum was set up in a standard manner (see e.g. Abramson, Barlow, Mustafa & Stephen-

son, 1969) in an apparatus which allowed five prepared drug solutions to be applied at regular intervals of 1.5 min in a predetermined order. The time of exposure to the agonists plus antagonist(s) was 15 seconds. Two separate sets of experiments were performed, each experiment being on a different piece of ileum. In one set (20 experiments), all the solutions contained only one antagonist, S (see *Notation*), in a concentration of 6.28×10^{-10} M. The agonist solutions contained A in a concentration of either $[A] = 7.2 \times 10^{-5}$ M or $[A]' = 2.16 \times 10^{-4}$ M, or C in a concentration of 5×10^{-6} M or 1×10^{-5} M. In the other set (9 experiments), all the solutions contained both antagonists: S, in the same concentration as above and F in a concentration of 1.89×10^{-4} M. The agonist solutions contained either [A] or [A]', or C in a concentration of 1.6×10^{-4} M or 3.2×10^{-5} M. The concentrations of C required to match those of A were calculated for each experiment by analyzing it as if it were two simultaneous 2 + 1 assays. Thus each experiment produced values either for $[C]_1$ and $[C]'_1$ or for $[C]_2$ and $[C]'_2$.

An assumption made in the theoretical section is that the receptors are in equilibrium with S and F before each exposure to the agonist. This is not strictly true since S and F will be 'displaced' during exposure to the agonists and some time will be required for equilibrium to be restored after the agonist is washed out. The effect due to C should be negligible, for it occupies an insignificant proportion of the receptors; that due to A was minimized by applying it only once for every 8 applications of C, in a sequence of alternating high and low doses.

Methods of computation

Solution of the differential equations: diffusion ignored. The solutions to appropriate pairs of equations (for the presence of a single antagonist) were computed in the same way as described by Colquhoun (1968). The sets of three equations were solved initially by an eigenvalue method (see e.g. Colquhoun, 1968, p. 153) but later in a faster way which may be sketched as follows. Consider taking the Laplace transform of equation (1), and solving, formally in terms of the Laplace parameter s for $\mathcal{L}(p)$ the agonist occupancy transform. Then $\mathcal{L}(p)$ will have the form $F(s)/G(s)$ where $F(s) = as^2 + bs + c$, and $G(s) = ds^3 + es^2 + fs + g$. The coefficients a, b, . . . g are easily found from the time constants, affinity constants and concentrations. Thus if r_1, r_2, r_3 are the roots of the equation $G(s) = 0$, the inverse of $\mathcal{L}(p)$ is

$$p = -\frac{F(0)}{r_1 r_2 r_3} + \frac{F(r_1)e^{r_1 T}}{r_1(r_1 - r_2)(r_1 - r_3)}$$

$$+ \frac{F(r_2)e^{r_2 T}}{r_2(r_2 - r_1)(r_2 - r_3)} + \frac{F(r_3)e^{r_3 T}}{r_3(r_3 - r_1)(r_3 - r_2)}$$

where T is the time of exposure to the agonist.

The accuracy of the computations was tested by comparing the results obtained for a slow antagonist with a very long time constant (10^6 times exposure time) and an agonist and fast antagonist with very short time constant (10^{-6} times exposure time) with the results given by direct calculation for an infinitely slow antagonist and an agonist and fast antagonist which reached equilibrium instantaneously (see appendix). The two results agreed to better than 1 in 10^4.

The curves showing p as a function of normalized concentration were drawn automatically on an XY plotter linked to the computer.

Diffusion of agonist taken into account. It is assumed above that the concentration of agonist rises to its final value instantaneously. Values were also computed on the assumption that the concentration at the receptors approached its final value exponentially i.e. according to $c_A(t) = c_A\{1 - \exp(-t/\tau_d)\}$ where τ_d is a time constant for diffusion. The modified equations were solved numerically either with the IBM programme for simultaneous differential equations, DHPCG (Scientific and Statistical Package) or with a faster procedure kindly made available by Mrs Joyce Acheson of the Biochemistry Department, University of Edinburgh. Agreement between the two methods was better than 1 in 10^4.

Results

Matching concentrations of the high and low efficacy agonists in the presence of one antagonist

Table 1 shows the concentrations $[C]_1$ and $[C]'_1$ of the high efficacy agonist, pentyl TMA (see notation), which in the presence of the slow antagonist, benzilyltropine methiodide, were found to match the two concentrations [A] and [A]' of the low efficacy agonist, hexyl TMA. The ratio $[A]'/[A]$ was 3.0; the ratio of the mean values of the matching concentrations, $[C]'_1/[C]_1$ was 1.294. The occupancies corresponding to $[C]_1$ and $[C]'_1$ were calculated by the methods outlined in the *Theoretical* section from the normalized concentrations of the agonist and antagonist for various assumed values of their dissociation time

constants. K_S was taken as 2.36×10^{10} M^{-1} (Barlow & Mustafa, 1968) and K_C was taken as 5.37×10^3 M^{-1} (Abramson *et al.*, 1969). Figure 3 shows that the calculated occupancies p_1 and p_1' respectively were not greatly affected by the value assumed for the dissociation time constant of the slow antagonist. They were of course markedly affected by the value chosen for τ_C, the dissociation time constant of the agonist, pentyl TMA (Figure 4). Their ratio p_1'/p_1, however, lay between 1.28 and 1.30 whatever values were assumed for the time constants (Table 2). The insensitivity of the value of p_1'/p_1 to the various assumptions is a consequence of the high efficacy of the agonist pentyl TMA and the resulting low values of the matching normalized concentrations. This makes the occupancy ratio close to the ratio of the concentrations themselves as is shown in the Appendix, and demonstrates that pentyl TMA is a suitable choice for our comparison with the test agonist (see p. 289). As an indication of experimental error, the s.e. of the mean of the ratios of the matching concentrations was about 0.02. We have therefore assumed that the ratio p_1'/p_1 lies between 1.24 and 1.33.

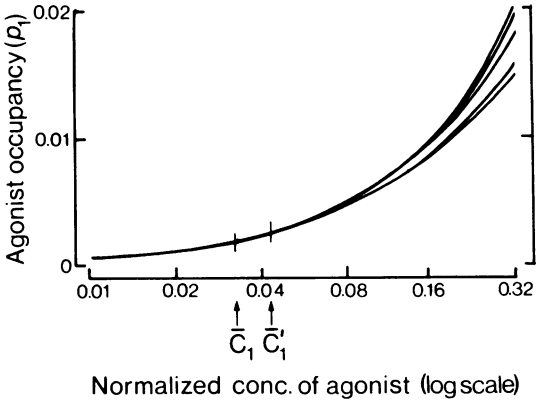

Fig. 3 Effect of dissociation time constant, τ_S, of slow antagonist, S, on relationship between 'high efficacy' agonist occupancy, p_1 and the concentration of agonist in the presence of the concentration of slow antagonist used in the experiment. Reading downwards the curves correspond to values of τ_S/T of 0, 5, 10, 100, and ∞. The curves corresponding to the extreme values for τ_S show the relationships that would obtain if equilibrium with S were instantaneous ($\tau_S = 0$) or if S were an irreversible antagonist ($\tau_S = \infty$). The abscissae $\bar{C}_1$ and $\bar{C}_1'$ correspond to the mean concentrations of pentyltrimethyl ammonium (high efficacy agonist) found to match the two concentrations of hexyltrimethyl ammonium (low efficacy agonist) that were used throughout the experiments. The agonist was assumed to equilibrate rapidly ($\tau_C = 0.001T$).

Table 1 Molar concentration ($\times 10^6$) of pentyltrimethyl ammonium bromide (pentyl TMA) $[C]_1$ which matched $[A] = 7.2 \times 10^{-5}$ M; and $[C]_1'$ which matched $[A]' = 2.16 \times 10^{-4}$ M hexyl TMA in the presence of 6.28×10^{-10} M benzilyltropine methiodide

Experiment number	$[C_1]$	$[C_1']$
1	6.57	8.04
2	5.98	8.20
3	6.57	8.26
4	5.81	8.04
5	5.95	7.55
6	6.26	8.48
7	6.23	7.42
8	5.78	7.67
9	5.83	7.24
10	5.88	6.74
11	6.44	9.02
12	5.24	7.01
13	5.24	6.11
14	5.79	8.33
15	5.48	6.80
16	5.28	6.38
17	5.69	7.55
18	6.43	9.86
19	6.01	7.39
20	6.85	8.58
mean	5.97	7.73

Table 2 Ratio of occupancies corresponding to the estimated means of the concentrations $[C]_1$ and $[C]_1'$ of pentyltrimethyl ammonium bromide (pentyl TMA) (see Table 1)

τ_d (s)	τ_s (min)	τ_c (s)	*Ratio* (p_1'/p_1)
0	∞	0	1.283
0	150	0.5	1.283
1	100	1.0	1.283
5	50	1.0	1.284
5	10	5.0	1.292
5	10	15.0	1.292
0	0	0	1.294

Various values for the time constants have been assumed. τ_d is the 'diffusion' time constant (concentration is assumed to rise to final value according to $C_1(t) = C_1(1 - e^{-t/\tau_d})$); τ_S is the dissociation time constant for benzilyltropine methiodide. τ_C is the dissociation time constant for pentyl TMA. The time of exposure to the agonist was 15 seconds. The first and last ratios were calculated as described in the appendix (p. 296): they correspond to (1) the 'ideal' situation in which the slow antagonist is effectively irreversible during exposure to the agonist and (2) the situation in which there is complete equilibration; they appear to be the limiting values.

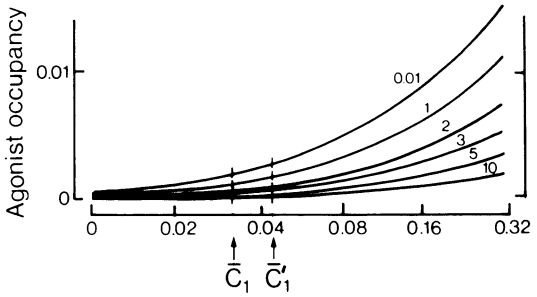

Fig. 4 Effect of dissociation time constant, τ_C, of 'high efficacy' agonist on occupancy *v.* concentration relationship in the presence of a slow antagonist (see caption to Figure 3). The numbers attached to each curve indicate the ratio of τ_C to the time of exposure to the agonist. The dissociation time constant for the antagonist was taken as 100T.

Matching concentrations of pentyltrimethyl ammonium bromide (pentyl TMA) in the presence of two antagonists and the corresponding occupancies

Table 3 shows the results from 9 experiments in which the concentrations $[C]_2$ and $[C]'_2$ of pentyl TMA which matched the two standard concentrations [A] and [A]′ in the presence of the original concentration of benzilyl tropine methiodide together with the second antagonist, pentyltriethyl ammonium (pentyl TEA). (Note the distinction between pentyl TMA (the high efficacy agonist) and pentyl TEA (the fast antagonist)). As before, the occupancies p_2 and p'_2 corresponding to the mean values $[C]_2$ and $[C]'_2$ may be calculated from their normalized concentrations and those of the antagonists, and from their dissociation time constants. The affinity constant K_F for pentyl TEA was taken as 3.58×10^4 M^{-1} (Abramson *et al.*, 1969). Figure 5 illustrates the relationship

Table 3 Molar concentrations ($\times 10^6$) of pentyltrimethyl ammonium bromide (pentyl TMA) $[C]_2$ which matched [A] = 7.2×10^{-5} M hexyl TMA and $[C]'_2$ which matched [A]′ = 2.16×10^{-4} M hexyl TMA, in the presence of 6.28×10^{-10} M benzilyltropine methiodide and 1.89×10^{-4} M pentyltriethyl ammonium bromide (see Table 1)

Experiment number	$[C]_2$	$[C]'_2$
21	13.6	31.5
22	14.5	31.9
23	13.0	30.0
24	14.6	32.3
25	14.7	31.9
26	13.2	30.6
27	13.9	30.8
28	14.0	32.7
29	13.6	30.2
mean	13.9	31.3

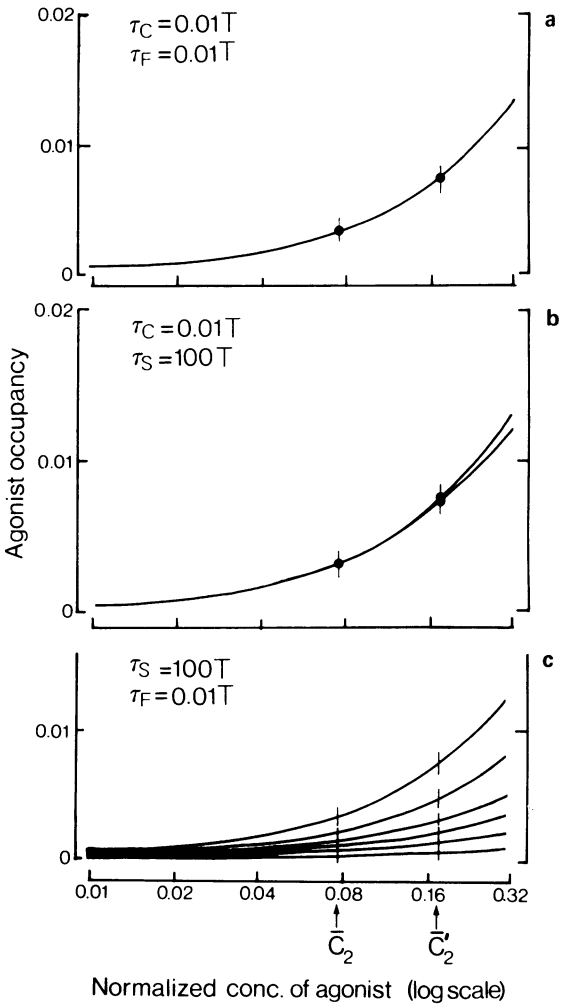

Fig. 5 Agonist occupancy *v.* concentration of agonist (abscissae) in presence of experimental concentrations of slow and fast antagonists under different assumptions about their time constants in (a) and (b) and that of the agonist in (c). The values $\bar{C}_2$ and $\bar{C}'_2$ correspond to the mean values of the experimental concentrations of the high efficacy agonist which matched the standard concentrations of hexyltrimethyl ammonium (see Table 3).

Six superimposed curves, reading downwards: (a) τ_S/T is 0, 1, 5, 10, 100, and ∞; (b) τ_S/T is 0.01, 0.1, 0.5, 1, 2 (these merge) and 5; (c) τ_C/T is 0.01, 1, 2, 3, 5 and 10.

between agonist occupancy and concentration with various assumptions about the dissociation time constants. As was the case with a single antagonist it again happens that within wide limits the values assumed for the dissociation time constants for the antagonists have a negligible effect on the values of the occupancies (Figure 5, a and b). The value of the dissociation time constant τ_C, as was also the case with a single antagonist, does affect the value of the occupancies (Figure 5, c). But, as before, it is not the individual occupancies, p_2 and p'_2 that are required for the testing of the hypothesis. What are required are the ratios $R = p_2/p_1$ and $R' = p'_2/p'_1$. A selection of estimates for these ratios calculated with various different assumptions is shown in Table 4.

In summary, they point to a value of between 1.6 and 1.7 for R and between 2.8 and 2.9 for R'. Taking experimental error into account, we have assumed that the ratio R lies between 1.5 and 1.8 and that R' lies between 2.7 and 3.0.

Effect of errors in the value of the affinity constant of the high efficacy agonist

Additional calculations were made to see to what extent the occupancy ratios deduced from the matching concentrations of the high efficacy agonist would be affected if an inaccurate value had been used for its affinity constant. An overestimate for K_C by a factor of 2 would have been without effect; an underestimate by a factor of 2 would have produced a change of less than 1% provided that τ_S was greater than 25 minutes.

Table 4 Ratios p_2/p_1 and p'_2/p'_1 of occupancy by concentrations $[C]_2$ and $[C]'_2$ of agonist in presence of two antagonists, S and F to occupancy by concentrations $[C]_1$ and $[C]'_1$ in the presence of one antagonist, S (see Tables 1 and 3)

τ_d (s)	τ_S (min)	τ_C (s)	τ_F (s)	p_2/p_1	p'_2/p'_1
0	∞	0	0	1.667	2.891
1	150	1.0	0.5	1.666	2.890
5	150	1.0	1.0	1.663	2.885
5	50	1.0	5.0	1.661	2.878
5	10	1.0	5.0	1.654	2.864
5	10	5.0	15.0	1.641	2.826
0	0	0	0	1.629	2.823

For definition of τ_d, τ_S and τ_C see footnote to Table 2. τ_F is the dissociation time constant for the fast antagonist, pentyltriethyl ammonium. The ratios in the first and last lines were calculated as described in the appendix (p. 298). They appear to represent limiting values.

Predicted values for the occupancy ratios for hexyltrimethyl ammonium

We have now obtained values for three independent ratios of occupancies by pentyl TMA, p_1/p_1, R and R' which, as discussed in the theoretical section (p. 289), should be the same as the ratios $p'_A(S)/p_A(S)$, $p_A(S, F)/p_A(S)$ and $p'_A(S, F)/p'_A(S)$ for hexyl TMA.

Before considering detailed values for K_A, τ_A, τ_S and τ_F, it is of interest to see how far the results discriminate between the two limiting conditions:

(a) the ideal situation for the present hypothesis in which the slow antagonist is effectively irreversible during exposure to the agonist but nonetheless it and the fast antagonist equilibrate with the receptors completely;

(b) the situation usually considered, in which equilibrium between agonist, both antagonists and the receptors is complete within the period of exposure to the agonist.

The results of calculation made as described in the appendix, equations (i), (ii), (v) and (vi), on these two sets of assumptions are shown in Table 5. If K_A is between 2.7 and 3.2×10^4 M^{-1} the three predicted values for the ratios are within the range of the experimental values at the limiting conditions of the present hypothesis. By contrast, there is an enormous discrepancy over the whole range for K_A shown, 1.5 to 4.5×10^4 M^{-1}, if it is supposed that equilibrium is reached between the receptors and all three drugs. This is not surprising since the observed potentiation would not occur in that situation.

In the more general case, numerical solutions to the mass action equations (see *Theoretical section*) were obtained for a range of assumed values for K_A (2.7×10^4 M^{-1} upwards), τ_A (0.5 s to 15.0 s), τ_S (10-150 min) and τ_F (0.5 s to 15.0 seconds). The lowest values of τ_A and τ_F yield solutions indistinguishable from those obtained if τ_A and τ_F are assumed to be zero. In one set of computations, diffusion was ignored; in another a highly simplified model was taken (see heading to Table 2) and it was assumed that the time constant for 'diffusion' of the agonist was 5 s (see also discussion section). The values for K_A, τ_A, τ_S and τ_F which predict values for the ratios $p'_A(S)/p_A(S)$, $p_A(S, F)/p_A(S)$ and $p'_A(S, F)/p'_A(S)$ in the observed range are shown in Tables 6, 7 and 8.

The results may be seen to be compatible with moderate departures from the 'ideal' conditions of an irreversible 'first' antagonist and an instantaneously equilibrating 'second' antagonist. An independent value for K_A of 1.8×10^4 M^{-1} has been obtained by one of us (Stephenson,

unpublished results); if the true value of K_A is fairly close to this, the first antagonist dissociates from the receptors considerably more slowly than the second, as postulated in the original hypothesis. Allowing that the value of K_A is not greater than 3.6×10^4 M^{-1}, the minimum value of τ_S (to the nearest minute) is 20 minutes. This assumes that τ_F and τ_A are close to zero. The maximum value of τ_F (which could occur if τ_S is greater than 80 min and τ_A less than 6 s) is 9 seconds.

Discussion

It will be appreciated that we have throughout adopted a particular view of drug-receptor interactions. An alternative framework is provided by various allosteric models (see e.g. Changeux & Podleski, 1968). However, at present it does not seem profitable to attempt an alternative analysis. Results of the kind described in this paper are extremely unlikely to discriminate between the 'classical' and 'allosteric' models (see Colquhoun, 1973; Thron, 1973). Another possibility which unfortunately cannot be excluded, is that the slow antagonist is not intrinsically slowly dissociating but is made to appear so by slow diffusion from a restricted region ('the biophase') in the neighbourhood of the receptors (see Furchgott, 1964; Thron & Waud, 1968; Colquhoun, Henderson & Ritchie, 1972). Suppose also that the amount of antagonist bound to the receptors is large in relation to the amount present in the biophase, on account of the high affinity of the (first) antagonist. When the agonist is added, although the dissociation of the antagonist is fast, the agonist occupancy is

Table 5 Theoretical values for the ratios $p'_A(S)/p_A(S)$, $p_A(S, F)/p_A(S)$ and $p'_A(S, F)/p'_A(S)$ for different assumed values of K_A under two different sets of assumptions: (a) the ideal conditions of an 'irreversible' and an 'instantaneous' antagonist ($\tau_S = \infty$, $\tau_F = 0$) and (b) both antagonists instantaneous ($\tau_S = 0$, $\tau_F = 0$).

	(a)			*(b)*		
K_A / 10^4 M^{-1}	$\frac{p'_A(S)}{p_A(S)}$	$\frac{p_A(S, F)}{p_A(S)}$	$\frac{p'_A(S, F)}{p'_A(S)}$	$\frac{p'_A(S)}{p_A(S)}$	$\frac{p_A(S, F)}{p_A(S)}$	$\frac{p'_A(S, F)}{p'_A(S)}$
1.5	1.47	1.28	2.10	2.66	0.71	0.74
1.6	1.45	1.31	2.16	2.64	0.71	0.74
1.7	1.43	1.35	2.22	2.62	0.72	0.74
1.8	1.41	1.38	2.28	2.61	0.72	0.74
1.9	1.39	1.41	2.34	2.59	0.72	0.75
2.0	1.38	1.44	2.39	2.57	0.72	0.75
2.1	1.36	1.47	2.45	2.55	0.72	0.75
2.2	1.35	1.50	2.50	2.54	0.72	0.75
2.3	1.34	1.53	2.55	2.52	0.72	0.75
2.4	1.32	1.56	2.60	2.51	0.72	0.76
2.5	1.31	1.59	2.64	2.49	0.72	0.76
2.6	1.30	1.62	2.69	2.48	0.72	0.76
2.7	1.29	1.65	2.73	2.46	0.72	0.76
2.8	1.28	1.68	2.78	2.45	0.72	0.76
2.9	1.28	1.70	2.82	2.43	0.73	0.77
3.0	1.27	1.73	2.86	2.42	0.73	0.77
3.1	1.26	1.76	2.89	2.41	0.73	0.77
3.2	1.25	1.78	2.93	2.39	0.73	0.77
3.3	1.25	1.81	2.97	2.38	0.73	0.77
3.4	1.24	1.84	3.00	2.37	0.73	0.77
3.5	1.23	1.86	3.04	2.35	0.73	0.78
3.6	1.23	1.89	3.07	2.34	0.73	0.78
3.7	1.22	1.91	3.10	2.33	0.73	0.78
3.8	1.22	1.94	3.14	2.32	0.73	0.78
3.9	1.21	1.96	3.17	2.31	0.73	0.78
4.0	1.21	1.98	3.20	2.29	0.73	0.78
4.1	1.20	2.01	3.23	2.28	0.74	0.78
4.2	1.20	2.03	3.25	2.27	0.74	0.79
4.3	1.19	2.05	3.28	2.26	0.74	0.79
4.4	1.19	2.07	3.31	2.25	0.74	0.79

The agonist is assumed to be instantaneous in both (a) and (b). The boxed values are those which fall within the experimentally determined ranges.

Table 6 Ranges of values calculated for τ_S, τ_A, τ_F, and K_A which predict values of $p'_A(S)/p_A(S)$ from 1.24 to 1.33, for $p_A(S, F)/p_A(S)$ from 1.5 to 1.8 and for $p'_A(S, F)/p'_A(S)$ from 2.7 to 3.0

τ_S (min)	τ_A (s)	τ_F (s)	K_A 10^4 M^{-1}
150	0-1	0-5	2.8-3.3
		10	3.2-3.4
	5	0-1	2.8-3.5
	10	0-1	3.3-3.6
100	0-1	0-5	2.9-3.4
		10	3.3-3.5
	5	0-1	2.9-3.5
		5	3.3-3.5
	10	0.5	3.3-3.7
		1	3.4-3.7
50	0-1	0-5	3.1-3.6
	0.5	10	3.5-3.6
	1	10	3.6
	5	0-1	3.1-3.7
		5	3.4-3.7
	10	0.5	3.5-3.9
		1	3.6-3.9
25	0-1	0-5	3.5-3.9
	5	0-1	3.5-4.0
		5	3.9-4.0
	10	0.5	3.9-4.3
		1	4.0-4.3
10	10	0.5	5.4-5.6
		1	5.5-5.6

The time required for diffusion of the agonist was neglected. The values tested were: τ_S, 10, 25, 50, 100 and 150 min; τ_A and τ_F, 0.5, 1, 5, 10 and 15 seconds.

Table 7 Ranges of values calculated for τ_s, τ_A, τ_F and K_A as in Table 6

τ_S (min)	τ_A (s)	τ_F (s)	K_A 10^4 M^{-1}
150	0.5	0.5	2.9-3.5
		1	3.0-3.5
	1	0-1	3.0-3.5
		5	3.1-3.6
	5	1	3.3-3.8
100	0-1	0-1	3.0-3.6
		5	3.2-3.7
	5	0.5	3.3-3.9
		1	3.4-3.9
	10	0-1	4.3-4.4
50	0-1	0.5	3.2-3.7
	0.5	1	3.2-3.7
	1	1	3.2-3.8
		5	3.4-3.8
	5	0-1	3.5-4.0
	10	0.5	4.3-4.6
		1	4.5-4.6
25	0-1	0-1	3.6-4.1
		5	3.9-4.2
	5	0-1	3.9-4.4

The concentration of the agonist was assumed to reach its steady concentration with a time constant of 5.0 s (see Table 2).

Table 8 Summary from Tables 6 and 7 of ranges (for different values of K_A) for τ_S, τ_A, and τ_F which allow values for the calculated occupancy ratios consistent with those inferred from the experimental results

	No diffusion			*Diffusion time constant = 5 s*		
K_A 10^4 M^{-1}	τ_S (min)	τ_A (s)	τ_F (s)	τ_S (min)	τ_A (s)	τ_F (s)
2.8	150	0-5	0-5	–	–	–
2.9	100-150	0-5	0-5	150	0.5	0.5
3.0	100-150	0-5	0-5	100-150	0-1	0-1
3.1	50-150	0-5	0-5	100-150	0-1	0-1
3.2	50-150	0-5	0-5	50-150	0-1	0-5
3.3	50-150	0-10	0-5	50-150	0-1	0-5
3.4	50-150	0-10	0-10	50-150	0-1	0-5
3.5	25-150	0-10	0-10	25-150	0-5	0-5
3.6	25-150	0-10	0-10	25-150	0-5	0-5
3.7	25-100	0-10	0-5	25-150	0-5	0-5
3.8	25-50	0-10	0-5	25-150	0-5	0-5
3.9	25-50	1-10	0-5	25-100	0-5	0-5
4.0	25	5-10	0-5	25-50	0-5	0-5
4.1	25	10	0-1	25-50	0-5	0-5
4.2	25	10	0-1	25	0-5	0-5
4.3	25	10	0-1	25-150	5-10	0-1
4.4	–	–	–	25-100	5-10	0-1

depressed by the increase in concentration of the antagonist in the biophase. After equilibration with a second antagonist of much lower affinity, the amount of the first antagonist bound to the receptors is smaller. The increase in its concentration during the presence of the agonist will therefore be smaller and so also will be the depression of the response to the agonist. It might be that the reduction in the depression caused by the accumulation of the first antagonist in the biophase outweighs the effect of the second antagonist, thus providing an alternative explanation for the paradoxical potentiation. It is, however, difficult to make quantitative predictions for such a model, and it seems preferable to summarize the results in terms of the original hypothesis.

If it is supposed that the value for K_A, the affinity constant for hexyltrimethyl ammonium, obtained by Stephenson (unpublished results) is in error by less than a factor of 2, (i.e. $K_A < 3.6 \times 10^4$ M^{-1}), the results suggest that the dissociation time-constant for the antagonist presumed to be slow is 20 min or more, whereas the dissociation time-constants for the 'fast' antagonist and the agonist are less than 10 seconds. Additional calculations have been made to see how these values would be affected if the published values of the affinity constants of the antagonists were in error. It was found that the conclusions were unmodified by changes in the affinity of the slow antagonist within a factor of 2. This is fortunate since, being slow to develop the antagonism is difficult to measure accurately. For the fast antagonist, if the value taken were an overestimate by a factor of 2, the discrepancies between the inferred and predicted values of the ratios of the occupancies would be so great that the hypothesis could be rejected. Since the affinity of fast acting antagonists is easy to measure this is unlikely. If the value taken were an underestimate by a factor of 2, the minimum dissociation time-constant for the slow antagonist would be about 10 min and, as before, the maximum for the other substances, about 10 seconds.

In summary, the results appear to be consistent with the initial hypothesis, and even allowing for considerable errors in the determination of the various affinity constants, require that the slow antagonist dissociates from the receptors at least 60 times more slowly than do either the fast antagonist or the agonist.

Appendix

(a) Limiting conditions

It may be of interest to see how the limiting conditions in which $\tau_S = \infty$, $\tau_A = 0$, $\tau_F = 0$ can be investigated without the necessity of solving differential equations. The conditions are equivalent to supposing that equilibrium between the receptors and the agonist, A, and fast antagonist, F, is attained within the period of exposure of the tissue to the agonist, but that dissociation of the slow antagonist, S, from the receptors is negligible during this period. The theory is used to derive the approximate minimum value of K_A from the observed matching concentrations (section b) and to calculate approximate values of the occupancy ratios from the matching concentrations of the high efficacy antagonist (section c).

Suppose that with the concentrations used, when each is present alone, the drugs A, S, and F, respectively, occupy proportions of receptors p_A, p_S, and p_F. The aim is to determine $p_A(S)$ and $p_A(S, F)$, the occupancy by the agonist in the presence, respectively, of S alone, and of S and F together, after equilibration with the receptors (see *Notation*).

Evidently,

$$p_A(S) = p_A(1 - p_S) \qquad \text{(i)}$$

To find $p_A(S, F)$ we note that the occupancies by S and F when both are present are given by:

$$p_F(S) = p_F(1 - p_S(F))$$

$$p_S(F) = p_S(1 - p_F(S))$$

whence

$$p_F(S) = \frac{p_F(1 - p_S)}{1 - p_F p_S}$$

and

$$p_S(F) = \frac{p_S(1 - p_F)}{1 - p_F p_S}$$

Now during exposure to the agonist, it equilibrates with the receptors to reach the occupancy $p_A(S, F)$ and the fast antagonist re-equilibrates to the occupancy $p_F(A, S)$. The occupancy of the slow antagonist remains $p_S(F)$. Thus,

$$p_A(S, F) = p_A\{1 - p_S(F) - p_F(A, S)\}$$

$$p_F(A, S) = p_F\{1 - p_S(F) - p_A(S, F)\}$$

whence

$$p_A(S, F) = \frac{p_A(1 - p_S)(1 - p_F)}{(1 - p_F p_A)(1 - p_F p_S)} \qquad \text{(ii)}$$

An equivalent expression is

$$p_A(S, F) = \frac{p_A(1 - p_S)(1 + c_F)}{\{c_F(1 - p_A) + 1\}\{c_F(1 - p_S) + 1\}} \qquad \text{(iii)}$$

where c_F is the normalized concentration of fast antagonist. The factor R by which the occupancy is changed, i.e. $p_A(S, F)/p_A(S)$ is given by

$$R = \frac{1 - p_F}{(1 - p_F p_A)(1 - p_F p_S)} \qquad \text{(iv)}$$

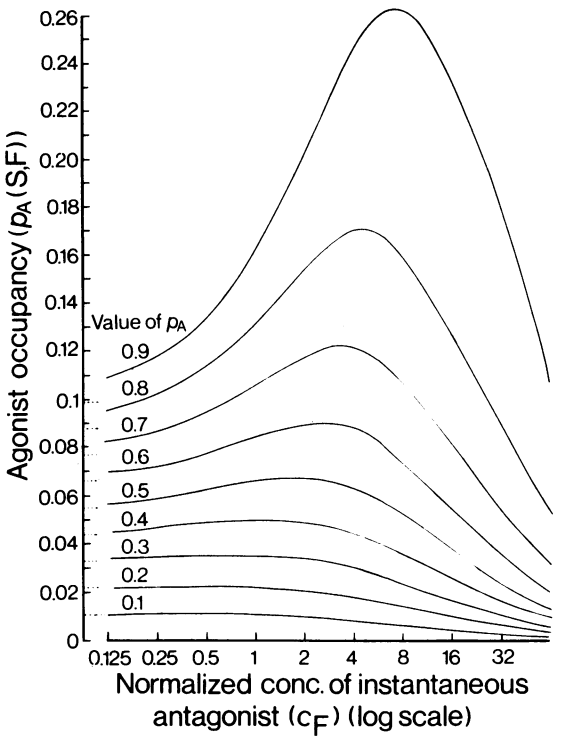

Fig. 6 Relationship between occupancy (ordinates, p_A(S, F)) by agonist, in presence of an infinitely slow antagonist which occupies 0.89 of the receptors, and concentration of instantaneous antagonist in normalized units. The relationship is shown for 9 different agonist concentrations which if present alone would have occupied 0.1 to 0.9 of the receptors (p_A).

If it is assumed that all three substances A, F, and S equilibrate instantaneously, then the formulae corresponding to (i) and (ii) may be shown to be:

$$p_A(S) = \frac{p_A(1-p_S)}{1-p_Ap_S} \qquad (v)$$

$$p_A(S, F) = \frac{p_A(1-p_S)(1-p_F)}{1+2p_Ap_Sp_F-p_Ap_S-p_Sp_F-p_Fp_A} \qquad (vi)$$

In this case, p_A(S, F) is always less than p_A(S) and R is of course less than one.

Figures 6 and 7 illustrate the way that p_A(S, F) and R vary with p_F and c_F respectively for different values of p_A and p_S from equations (iii) and (iv). For R to be greater than one, i.e. for the 'paradoxical' effect to occur, it can be shown as follows that $p_A + p_S$ must also be greater than one. For any given values p_A and p_S, the maximum value of R is obtained for a value p_F, p_F(max), such that

$$\frac{\partial R}{\partial p_F} = 0$$

whence

$$p_F(\max) = 1 - \sqrt{(p_Ap_S + 1 - p_A - p_S)/p_Ap_S}.$$

Evidently if $p_A + p_S < 1$, p_F(max) is negative, which is impossible; i.e. there is no maximum, and R decreases monotonically as p_F increases. The maximum value of R is given by

$$R(\max) = 1/\{p_A + p_S - 2[p_Ap_S - \sqrt{p_Ap_S(1-p_A)(1-p_S)}]\}$$

Interpretation of experimental results. Experimentally, suppose a concentration [A] of agonist A (efficacy e_A) is matched by $[C]_1$ of an agonist C of high efficacy, e, in the presence of [S] and by $[C]_2$ in the presence of [S] + [F]; and that $[A]'$ is matched by $[C]'_1$ and $[C]'_2$ respectively, in the presence of [S], and [S] + [F] (see *Notation*). If e is sufficiently large, p_1, p'_1 . . . may be taken as numerically equal to $K_C[C]_1$, $K_C[C]'_1$, . . .

(b) Limiting value of K_A

For equal responses to [A] and $[C]_1$ etc. (see Stephenson, 1956),

$$e_Ap_A(S) = ep_1$$

From (i),

$$e_Ap_A(1-p_S) = e(K_C[C]_1)(1-p_S)$$

Similarly,

$$e_Ap'_A(1-p_S) = e(K_C[C]'_1)(1-p_S)$$

whence

$$K_A = \frac{\dfrac{[A]'}{[A]} - \dfrac{[C]'_1}{[C]_1}}{[A]'\left\{\dfrac{[C]'_1}{[C]_1} - 1\right\}}$$

With the appropriate numerical values inserted,

$$K_A = \frac{3.0 - 1.294}{2.16 \times 0.294 \times 10^{-4}} = 2.69 \times 10^4 \text{ M}^{-1}$$

It may also be noted that $p'_1/p_1 = [C]'_1/[C]_1$, whence the approximate estimate of the standard error of the mean given on p.

(c) Limiting values of R from $[C]_1$ and $[C]_2$

The limiting values of R and R_1 may be calculated from (i) and (ii), taking p_1, p'_1, p_2 and p'_2 to be

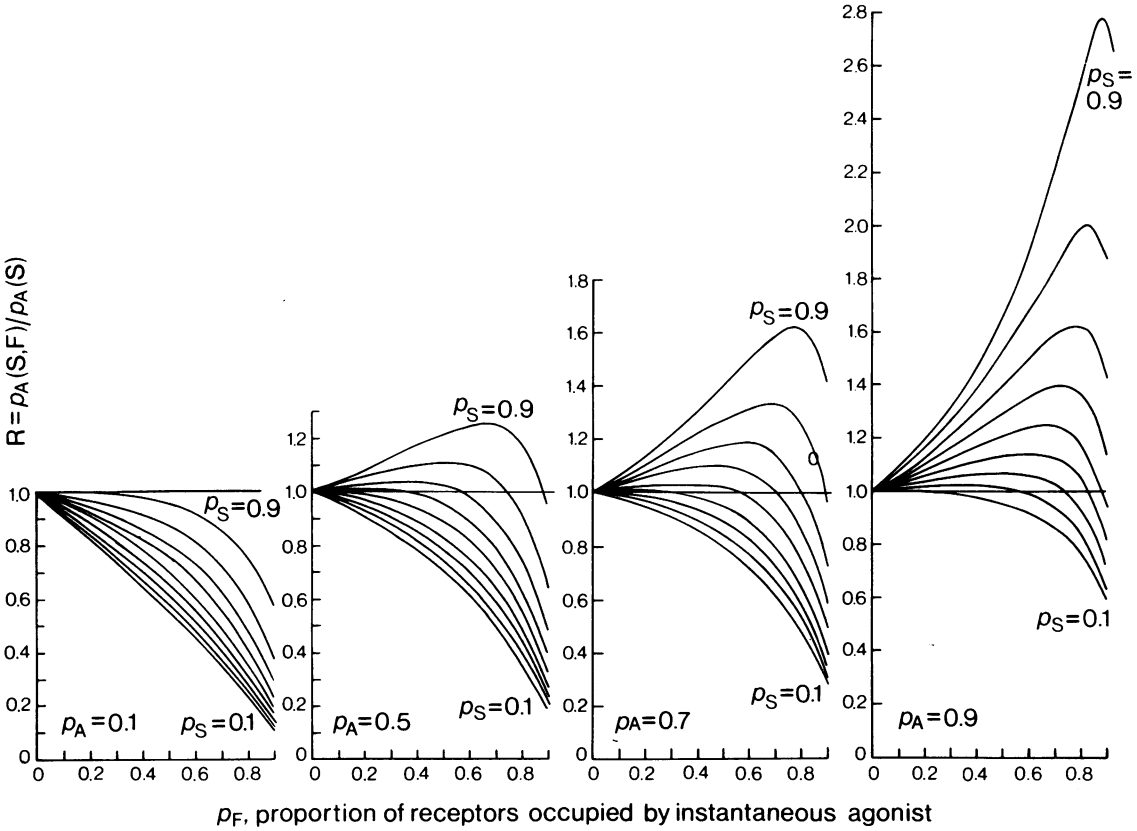

Fig. 7 Relationship between ratio, R of occupancy, p_A(S, F) by agonist in presence of 'irreversible' antagonist (which alone would occupy p_S) and instantaneous antagonist (which alone would occupy p_F) to that in presence of 'irreversible' antagonist alone, p_A(S) and p_F. In each panel, the agonist concentration is constant, p_A indicating what its occupancy would be if it were present alone.

negligible with respect to unity. Thus,

$$R = \frac{[C]_2}{[C]_1} \cdot \frac{1 - p_F}{1 - p_F p_S}$$

Inserting appropriate numerical values (p_S = 0.93679, p_F = 0.87124, and $[C]_1$, $[C]'_1$, $[C]_2$ and $[C]'_2$ from Tables 1 and 3),

$$R = 1.6314 \quad \text{and} \quad R' = 2.8372$$

These values are fairly close to those obtained by applying equations (i) and (ii) without approximation (see Table 4).

(d) Analogue computer solutions to equations

The value of plotting occupancy as a function of time has been illustrated by Paton & Waud (1964), Rang (1966) and Colquhoun (1968). Some insight into the present situation may be obtained by inspection of Fig. 8, which represents the superimposed solutions (drawn directly by an XY plotter linked to an analogue computer) to the equations governing combination of the agonist with its receptors in the absence of any antagonist and after equilibration with each antagonist separately and both together. For the particular concentrations shown (each of the drugs acting alone would occupy 90% of the receptors) and the relative time constants assumed (fast antagonist and agonist dissociate 100 times faster than the slow antagonist), 'paradoxical' potentiation would occur with an agonist exposure time even as long as 0.6 times the dissociation time constant of the slow agonist.

Figure 9 shows an analogue simulation of a possible paradoxical potentiation of junctional transmission. In (a) the supposed time course of the concentration of the agonist in the vicinity of the receptors is shown. The dissociation time

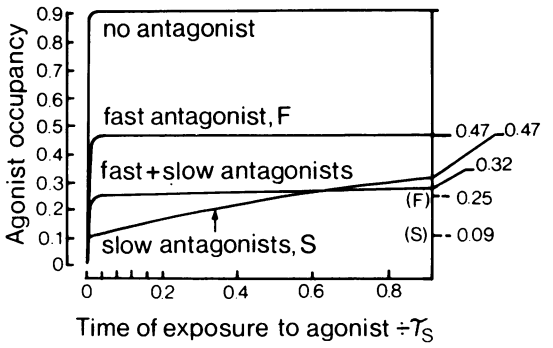

Fig. 8 Time course of agonist occupancy in presence of neither or one or both of the antagonists. Details of assumptions are given in the text. The marginal values are those equilibrium values which would be reached after indefinite exposure to the agonist (solid lines). The dashed lines show the values corresponding to an 'instantaneous' and irreversible antagonist. Each of the drugs if present alone would occupy 0.9. The dissociation time constants of agonist and fast antagonist were taken as 2×10^{-3} x that of the slow antagonist, τ_S.

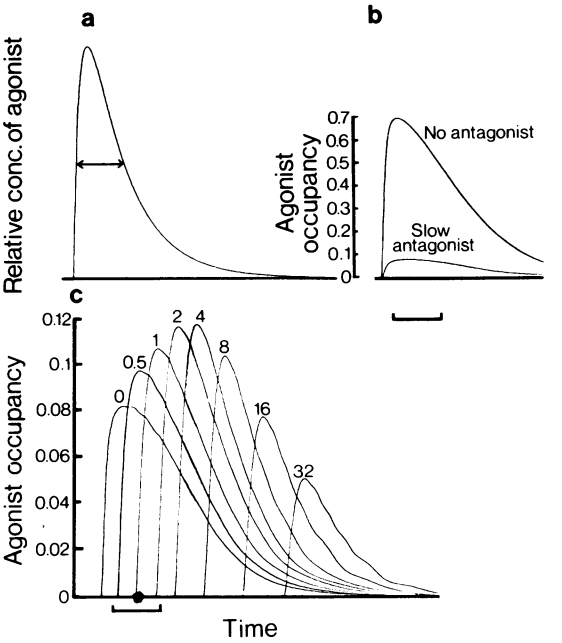

Fig. 9 Analogue computer simulation of the effect of slow and fast antagonists on occupancy of a transiently applied agonist. For details, see text. The horizontal bars represent the time for which the concentration of agonist is greater than half its maximum value. The 'oscillations' in (c) are an instrumental artifact.

constant of the agonist is taken to be 0.1 x T, T being the exposure half-time indicated in (a). In (b) the relationship between occupancy and time are shown, (1) in the absence of antagonist, and (2), after equilibration with a slow antagonist, its dissociation time constant being taken as 20 times τ, and in a concentration such that it occupies 0.89 of the receptors, before the agonist is applied. In (c) the effect of equilibration with a progressively increasing concentration of a fast antagonist, F, with a dissociation time constant of 0.1 times T added to the original slow antagonist, is shown. For clarity the curves have been displaced laterally; the displacement is arbitrary, each shift to the right corresponds to a doubling of the concentration of F, the normalized value c_F being indicated. It can be seen that as F increases there is initially a potentiation which eventually gives way to a further inhibition. An effect of this kind has been postulated by Ferry & Marshall (1973) to account for the interaction of tubocurarine and hexamethonium.

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