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Blockade by phenoxybenzamine of the contractor response produced by agonists in the isolated ileum of the guinea-pig

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Summary

1. The effects of various concentrations of phenoxybenzamine (dibenzyline) on the contractor response of the isolated ileum of the guinea-pig were investigated. The agonists tested were histamine, 5-hydroxytryptamine (5-HT), acetycholine and potassium chloride.

2. In addition, uptake of ^{14}C-phenoxybenzamine into the ileum was determined as a function of antagonist concentration. The uptake increases sharply at concentrations above 10^{-6} g/ml. (3×10^{-6}M) and was not saturable at any concentration tested.

3. In the presence of low concentrations of phenoxybenzamine, the dose-response curve for histamine undergoes a parallel shift of about 0·5 log units. At higher concentrations of phenoxybenzamine the maximum response is depressed. In the case of the other agonists, the maximum response is depressed as soon as any blockade becomes apparent.

4. The ease of blockade with phenoxybenzamine is 5-HT $\geq$ histamine $\gg$ acetylcholine $\geq$ potassium chloride.

5. These results do not lend support to the 'spare-receptor' hypothesis and may be better explained by the 'two-site' hypothesis of Moran & Triggle (1970).

6. It may further be concluded that the successful antagonism of potassium-induced contractions in this preparation lies in the ability of phenoxybenzamine to prevent the action of released acetylcholine. In the case of the contraction induced by 5-HT, phenoxybenzamine probably interferes with the 5-HT receptor responsible for neuronal release of acetycholine.

Introduction

Although the β-haloalkylamine class of drugs are generally regarded as blocking agents for α-adrenoceptors, they are also capable of inhibiting the responses to a wide variety of other agents (Furchgott, 1954). In particular the receptor system for histamine in intestinal smooth muscle is suceptible to blockade by these agents; however, the characteristic changes in the dose-response curves which result from this antagonism are not readily explicable. Nickerson (1956) reported that blockade with low doses of GD-121 caused a shift in the dose-response curve for histamine in isolated guinea-pig ileum, and that this shift was not accompanied by a decrease in the maximum response. A shift of two log units could be achieved, but at higher

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concentrations of antagonist the maximum response was depressed. This observation is not compatible with a one-to-one relationship between receptor occupancy and contractor response because β-haloalkylamines inactivate receptors in an irreversible manner. The proposed explanation was that only a small fraction of the available receptors need to interact with the agonist to elicit the maximum response; this hypothesis was referred to as the 'spare-receptor' or 'receptor-reserve' hypothesis.

The shifts in dose-response curve which occur in the histamine receptor system of guinea-pig ileum after blockade with GD-121 do not appear to have any analogies in the α-adrenoceptor system (May, Moran, Kimelberg & Triggle, 1967; Moran, Triggle & Triggle, 1969), nor is this behaviour characteristic of all receptor systems for histamine (Cook, 1970). There is, however, evidence for a receptor reserve for cholinomimetic compounds, in a variety of preparations (Ariëns, van Rossum & Koopman, 1960; Burgen, 1965; Furchgott & Bursztyn, 1967).

An alternative explanation for the unexpected shifts in dose-response curve sometimes obtained with irreversible antagonists, has recently been proposed by Moran & Triggle (1970), who used an alkylating analogue of a quaternary benzhydryl ether, and reported a shift of about 2 log units in the response to cholinergic agonists. Their studies also confirmed earlier reports by Ariëns *et al.* (1960) that no receptor reserve for partial agonists was demonstrable. Their evidence suggested that these results arose from the binding of the antagonist at more than one site. The initial binding may take place at the site of interaction of partial agonists, thus causing an immediate decrease in the response to these agents. Binding at this site was thought to introduce a small conformational change in the binding site for true agonists, thus necessitating higher doses of such agents, and hence a parallel shift in dose response curve. At higher concentrations the β-haloalkylamine was thought to interact with the site at which the cationic head of both true and partial agonists bind, thus causing a decrease in the maximum response to both types of agent.

In order to reinvestigate the 'spare-receptor' hypothesis in terms of both directly acting agents, and those which owe their activity to release of transmitter, the receptor systems for histamine, acetylcholine, 5-hydroxytryptamine (5-HT) and potassium ion were studied using the isolated guinea-pig ileum. Phenoxybenzamine was used as the antagonist, and the uptake of ^{14}C-phenoxybenzamine was also measured in this preparation to determine if there is any obvious correlation between the uptake of antagonist and blockade of different receptor systems.

Methods

The drugs used were histamine phosphate (Fisher Scientific), acetylcholine bromide (Eastman Organic) and 5-hydroxytryptamine creatinine phosphate (Nutritional Biochemicals). Phenoxybenzamine hydrochloride was synthesized by the method of Lewis & Miller (1966) and the 2-(N-phenoxyisopropylamino)-ethanol required in the synthesis was kindly donated by Smith, Kline & French. ^{14}C-Phenoxybenzamine was prepared from ^{14}C-benzylchloride (New England Nuclear) by a similar route. The product had a specific activity of 0·283 mCi/mmol. The infrared spectrum of the radioactive material was identical with that of an authentic sample of phenoxybenzamine and the mixed melting point was not depressed. The material was shown to be radiochemically pure, by the technique of isotope dilution. Doses of all drugs are

expressed as grammes of salt per millilitre of bathing medium; the approximate molar equivalent is also given, in parenthesis.

Adult male guinea-pigs weighing 200–400 g were killed by a blow on the head, and the ileum was excised and placed in a beaker containing Tyrode solution gassed with air (Edinburgh Staff, 1968). The contents of the ileum were removed by careful washing, and the preparation was cleaned of fat and connective tissue and cut into short segments 1–2 cm long. Four such segments were suspended in organ baths of 15 ml working volume, containing Tyrode solution at 37° C gassed with air. Contractions were recorded by means of force displacement transducers (Grass FTO3) connected to a polygraph (Grass P1).

In a typical experiment the tissues were allowed to equilibrate for 60 min, and the increase in tension produced by different concentrations of the agonist was recorded. The agonist was allowed to remain in contact with the tissue for 30 s and was washed out after each dose. An interval of 3 min was allowed after the tissue had returned to the resting tension before the administration of the next dose, except in the case of 5-HT where the interval was 6 minutes. The drugs were dissolved in normal saline, or, in the case of phenoxybenzamine, in normal saline containing 0·01 M hydrochloric acid, and 0·2 ml of an appropriate dilution was added to the organ baths to give the required concentration. The tissues were washed with Tyrode solution at intervals, and after 30 min, phenoxybenzamine hydrochloride was added. After the required exposure time (5 or 15 min) the tissues were washed twice, and thereafter at 15 min intervals for 120 minutes. A further dose-response curve for the agonist was then constructed. For the purpose of providing adequate controls, the experiment was repeated several times with omission of the blockade with phenoxybenzamine.

The uptake of ^{14}C-phenoxybenzamine was measured at different concentrations of the antagonist. Tissues which had been allowed to equilibrate under tension of 0·5 g in Tyrode solution for 60 min were exposed to ^{14}C-phenoxybenzamine in the required concentration, for 5 minutes. The tissues were washed using the procedure described earlier, removed from the bath, lightly blotted, and dried from the frozen state. Each tissue was weighed, transferred to a counting vial and dissolved in 5 M potassium hydroxide, by allowing the mixture to stand at room temperature overnight. Methanol (5·3 ml) was then added to each vial, and the total volume made up to 18 ml with scintillation fluid comprising 2,5-diphenyloxazole (PPO) (6·0 g) and *p*-bis-(2-[5-phenyloxazole])-benzene (POPOP) (0·1 g) dissolved in toluene (1 l.). The samples were stored in the dark for 2 days and counted for 40 min in two channels using a Nuclear Chicago Unilux scintillation counter, Model 6850. Corrections for quenching were made by means of the channels ratio method.

Results

To determine whether the response of isolated guinea-pig ileum changes during the course of the experiment, several control studies were carried out in which the blockade with phenoxybenzamine was omitted. The initial and final dose response curves obtained for the four agonists are shown in Figs. 1–4. The response is expressed in terms of the initial maximum response in the case of contractions induced by potassium chloride, and as a percentage of the initial response obtained at an agonist concentration of 10^{-6} g/ml (3×10^{-6}M) in the cases of histamine, 5-HT

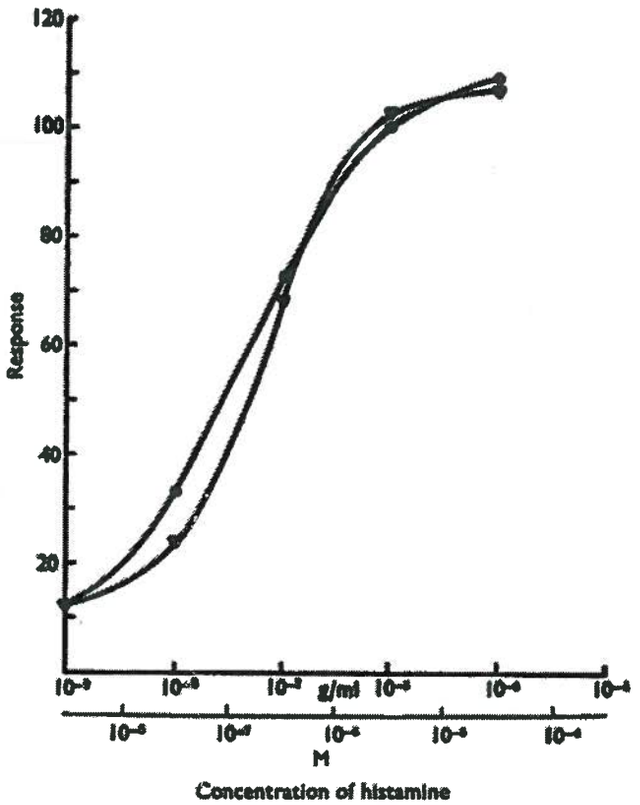

FIG. 1. Dose-response curves for histamine. ●——●, Initial response; ▼——▼, response after washing for 2 h in Tyrode solution. Response expressed as % initial response to 10^{-6} g/ml histamine. Each point represents the mean of at least twenty observations.

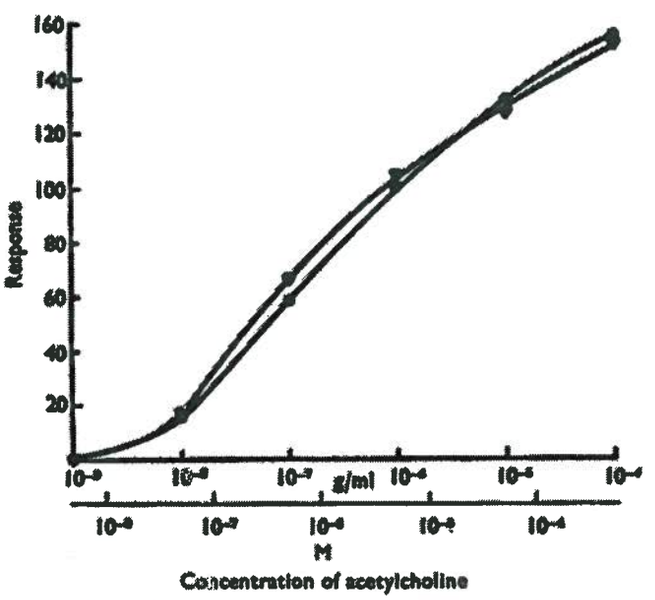

FIG. 2. Dose-response curves for acetylcholine. ●——●, Initial response; ▼——▼, response after washing for 2 h in Tyrode solution. Response expressed as % initial response to 10^{-6} g/ml acetylcholine. Each point represents the mean of at least twelve observations.

and acetylcholine. The latter technique for expressing contractility was used by Nickerson (1956) in his initial studies of the antagonism of the histamine response with GD-121, and has been used here to enable a comparison with these studies to be made. It can be seen from Figs. 1–4 that the responses do not change greatly during the course of the experiment; there is a small increase in the slope of the dose-response curve for histamine, and some change in shape of the curve for 5-HT. It should be pointed out, however, that it is more difficult to obtain a reproducible dose-response curve for 5-HT than for the other agonists used; the standard errors of each point are greater. In all cases the final dose-response curve was taken as the control for experiments in which blockade was examined.

The dose-response curves for histamine in the presence of various concentrations of phenoxybenzamine are shown in Fig. 5. At a phenoxybenzamine concentration 2×10^{-7} g/ml ($5{\cdot}9\times10^{-7}$M) and an exposure time of 5 min, a shift of about 0·5 log units is observed without depression of the maximum response. At higher concentrations the maximum response is depressed, and at concentrations of phenoxybenzamine of 5×10^{-6} g/ml ($1{\cdot}5\times10^{-5}$M) and higher, the response is abolished. The decrease in maximum response at a concentration of 5×10^{-7} g/ml ($1{\cdot}5\times10^{-6}$M) was significant at the 5% level.

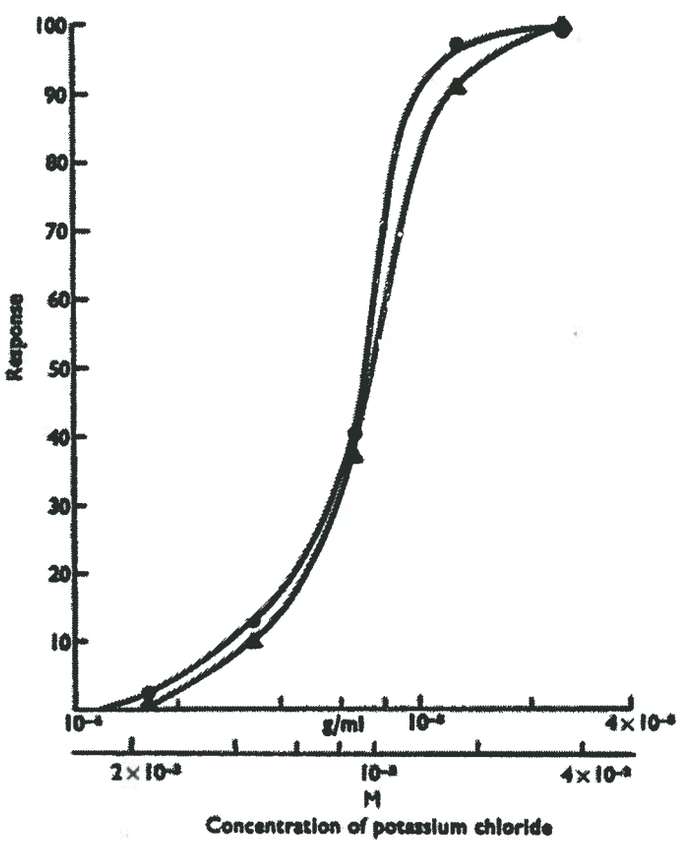

FIG. 3. Dose-response curves for potassium chloride. ▲——▲, Initial response; ●——●, response after washing for 2 h in Tyrode solution. Response expressed as % initial maximum response. Each point represents the mean of at least twelve observations.

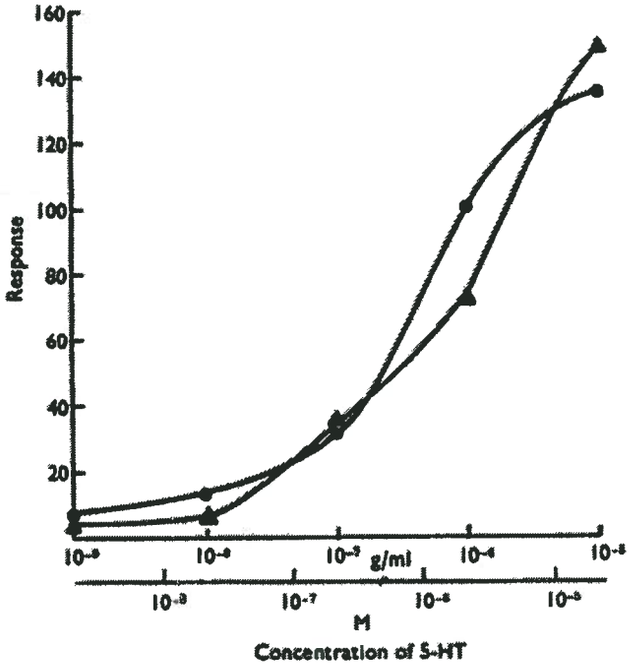

FIG. 4. Dose-response curves for 5-HT. ●—●, Initial response; ▲—▲, response after washing for 2 h in Tyrode solution. Response expressed as % initial response to 10^{-6} g/ml 5-HT. Each point represents the mean of at least twelve observations.

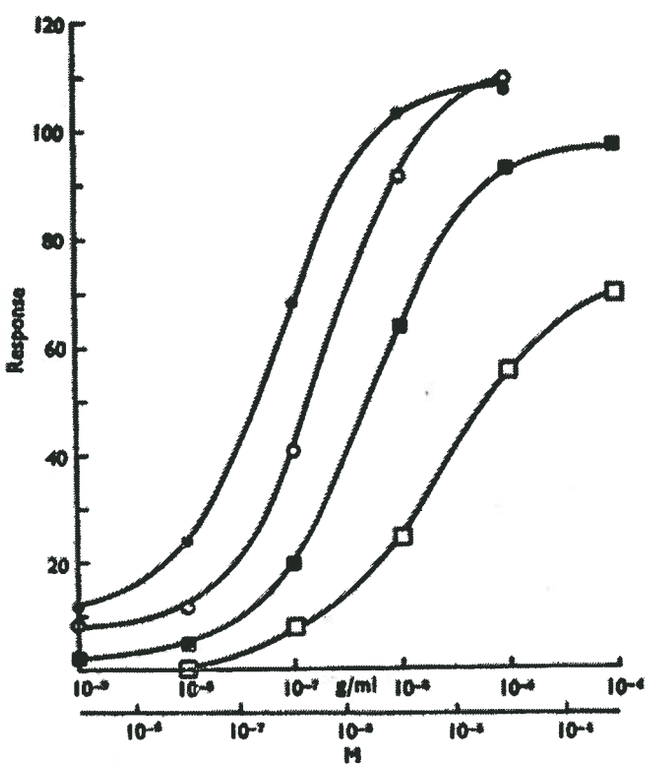

FIG. 5. Responses to histamine in the presence of various concentrations of phenoxybenzamine. ●—●, Control; ○—○, $2{\cdot}5\times10^{-7}$ g/ml; ■—■, 5×10^{-7} g/ml, and □—□, 10^{-6} g/ml. Response expressed as % initial response to 10^{-6} g/ml histamine. Each point represents the mean of at least twelve observations.

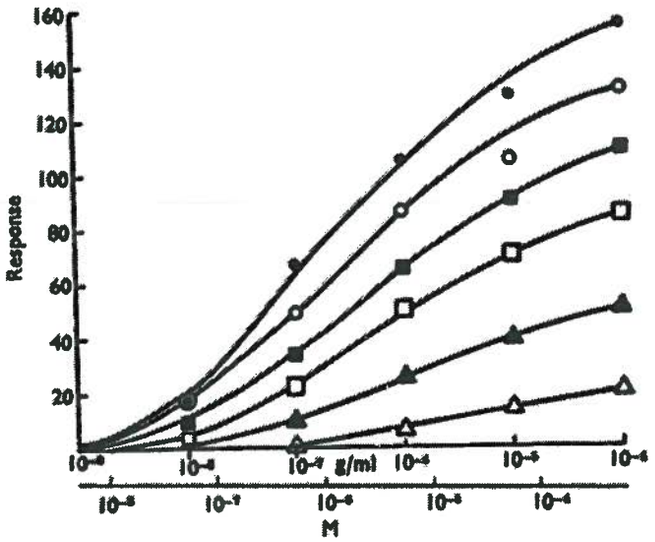

FIG. 6. Responses to acetylcholine in the presence of various concentrations of phenoxybenzamine. ●——●, Control; ○——○, 10^{-6} g/ml; ■——■, 5×10^{-6} g/ml; □——□, 10^{-5} g/ml; ▲——▲, 10^{-4} g/ml; △——△, 10^{-4} g/ml with exposure time increased to 15 minutes. Response expressed as % initial response to 10^{-6} g/ml acetylcholine. Each point represents the mean of at least twelve observations.

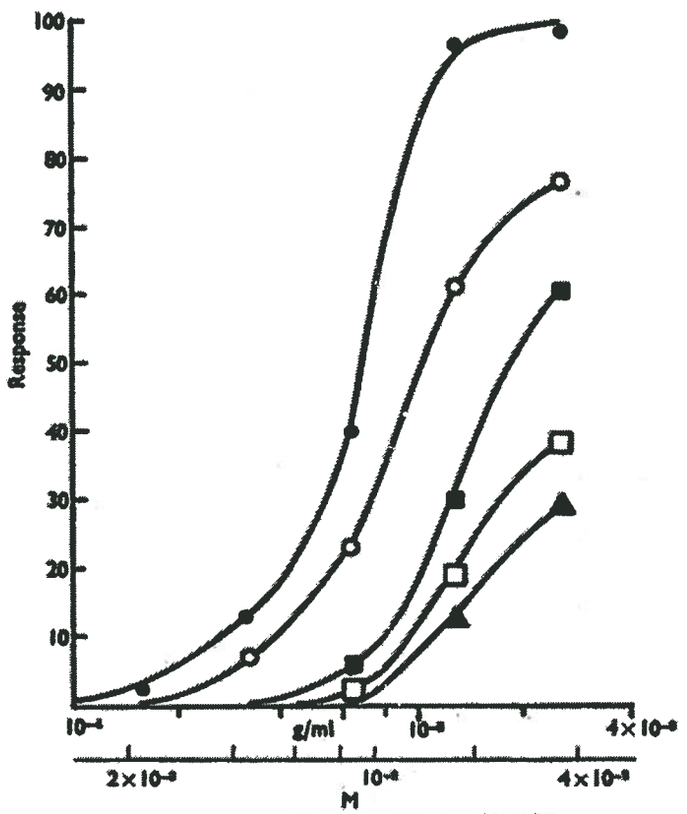

FIG. 7. Responses to potassium chloride in the presence of various concentrations of phenoxybenzamine. ●——●, Control; ○——○, 10^{-6} g/ml; ■——■, 10^{-5} g/ml; □——□, 10^{-4} g/ml; ▲——▲, 10^{-4} g/ml with exposure time increased to 15 minutes. Response expressed as % initial maximum response. Each point represents the mean of at least twelve observations.

Similar dose-response curves for acetylcholine are shown in Fig. 6. Blockade does not become apparent until a phenoxybenzamine concentration of 10^{-8} g/ml (3×10^{-8}M) is used. The progressive blockade produced at higher concentrations reduces the maximum response, and no parallel shift is observed in this case. A concentration of 10^{-4} g/ml (3×10^{-4}M) and an exposure time of 5 min does not abolish the acetylcholine-induced contraction. It is not possible to investigate the effects of higher doses of antagonist, since at the pH of the Tyrode solution, the antagonist precipitates at such concentrations. The exposure time was thus extended to 15 min, and under these conditions the response was further reduced, but still not abolished.

It may be seen from Fig. 7 that the responses to potassium are influenced by phenoxybenzamine in much the same way as those to acetylcholine. There is no parallel shift in dose-response curve, but at high concentrations of phenoxybenzamine the response to potassium is more resistant to blockade. The responses to 5-HT (Fig. 8) are much more sensitive to phenoxybenzamine than those of acetylcholine or potassium. The maximum response to 5-HT is achieved with an agonist concentration of 10^{-8} g/ml ($2{\cdot}5\times10^{-8}$M) in both blocked and unblocked preparations; higher concentrations cause a diminution of the response. Blockade with phenoxybenzamine at a concentration of 10^{-7} g/ml (3×10^{-7}M) causes a small decrease in the maximum response, and the response was abolished by concentrations of phenoxybenzamine of 5×10^{-6} g/ml ($1{\cdot}5\times10^{-5}$M) and higher. Thus it would appear that the maximum response is depressed as soon as any blockade becomes apparent in the receptor system for 5-HT.

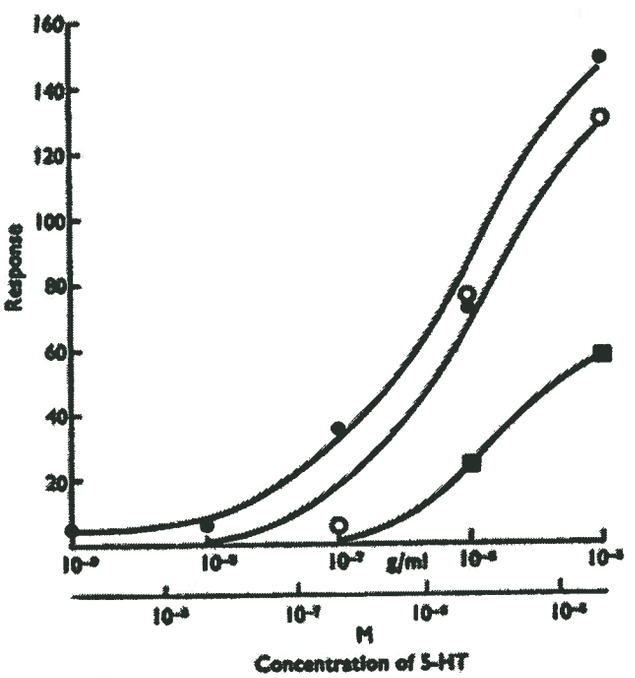

FIG. 8. Responses to 5-HT in the presence of various concentrations of phenoxybenzamine. ●—●, Control; ○—○, 10^{-7}; ■—■, 10^{-6}. Response expressed as % initial response to 10^{-6} g/ml 5-HT. Each point represents the mean of at least twelve observations.

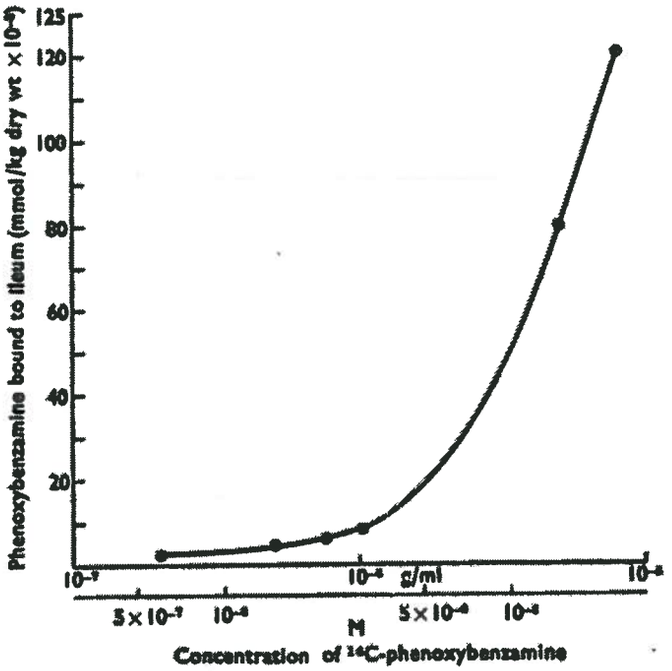

FIG. 9. Uptake of ^{14}C-phenoxybenzamine as a function of concentration.

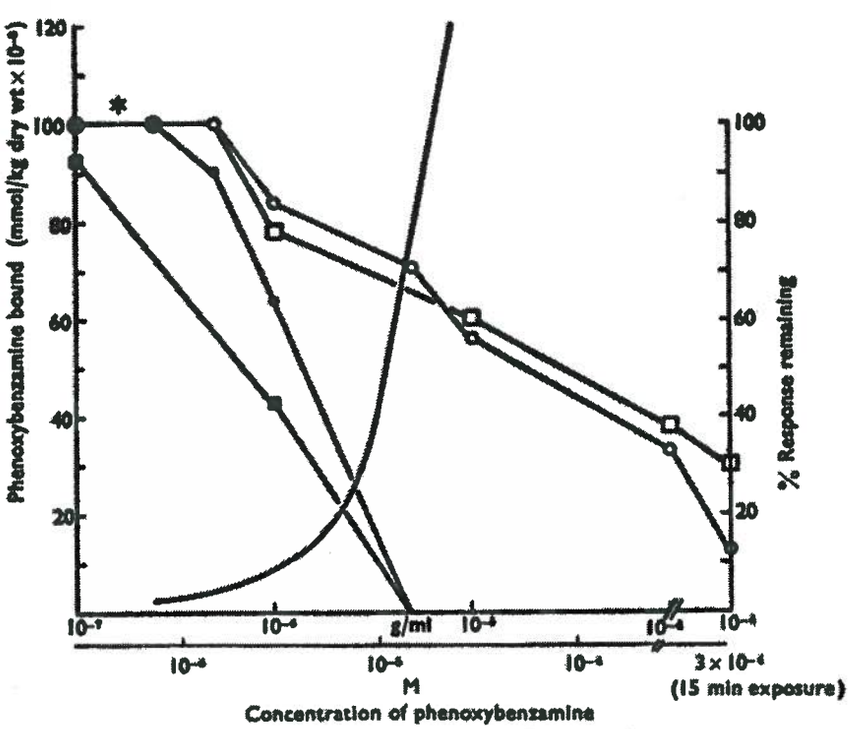

FIG. 10. Uptake of ^{14}C-phenoxybenzamine (continuous line) and the diminution in maximum response achieved with various concentrations of phenoxybenzamine. The diminution in response is expressed as a % of the control value. ●—●, Histamine; (○—○, acetylcholine; ■—■, 5-HT; □—□, potassium chloride. * Indicates that over this concentration range, phenoxybenzamine produces a shift in the dose-response curve for histamine without diminishing the maximum response.

The uptake curve of ^{14}C-phenoxybenzamine is shown in Fig. 9. The uptake at low concentrations is small and approximately linear. At concentrations above 10^{-6} g/ml (3×10^{-6}M) the uptake of ^{14}C-phenoxybenzamine increases sharply. It is interesting to note that this curve is closely similar to that obtained by Yong & Marks (1967) for ^{14}C-dibenamine. Figure 10 represents a summary of the data; the uptake together with the percentage of the maximum response obtained at different concentrations of phenoxybenzamine is shown for each agonist.

Discussion

Examining first the effects of phenoxybenzamine on histamine-induced contractions, the shift in the dose-response curve may be taken as evidence for a small receptor reserve. The magnitude of such a receptor reserve would, however, be much less than that observed in the studies quoted previously which used GD-121. It may be seen from Fig. 5 that the maximum shift which could be obtained in this system is less than one log unit, since such a curve would overlap with that obtained at an antagonist concentration of 5×10^{-7} g/ml ($1{\cdot}5\times10^{-6}$M) in which the response is depressed. Since the 'spare-receptor' hypothesis suggests that the observed shift in the dose-response curve is a function of the percentage of spare receptors, and thus independent of the antagonist used, the discrepancy between the results reported here, and those obtained with GD-121 indicate that an alternative hypothesis should be considered. It is appropriate to mention, however, before further discussion of these results that experiments involving β-haloalkylamines for evaluation of the spare receptor concept are open to one objection on methodological grounds. It has been suggested by Waud (1968) that the phenomenon may arise not from blockade of spare receptors, but from blockade of 'spare cells'. If it is supposed that only a fraction of the muscle cells need to contract in order to achieve maximum isotonic shortening, blockade of the readily accessible cells will provide a parallel shift in the dose-response curve to the agonist. A further consequence of this suggestion is that isometric measurements such as those used here might provide different results from the isotonic measurements commonly used in other studies of this type. If this suggestion is correct, the presence or absence of a parallel shift in dose-response curve after irreversible blockade becomes a function of factors such as duration of exposure to antagonist, tissue thickness or method of measurement, rather than of receptor occupancy. In these circumstances it would be anticipated that such shifts would occasionally be encountered for all receptor systems, and would depend critically on the exact method of measurement; in particular, the shorter the time of exposure to the antagonist, the more pronounced should be the shift. While there is insufficient evidence to evaluate this hypothesis in terms of the criteria mentioned above, it would seem that data currently available do not support these suggestions. Further work will be necessary before any definite conclusions can be drawn as to the existence of this mechanism.

If the parallel shift in dose-response curve arises from a phenomenon related to the characteristics or number of receptors rather than a mechanism of the type discussed above, it is necessary to consider alternative hypotheses to the simple 'receptor-reserve' concept, for reasons outlined at the begininng of this discussion. At least three such hypotheses may be formulated. First, it is possible that both sets of results arise, not from inactivation of receptors, but from a prolonged competitive

phase of action which is more pronounced in the case of GD-121. This explanation has been used by Moran *et al.* (1969) to dispute the existence of a receptor reserve in rat vas deferens. It is, however, unlikely to apply in this case; no evidence for a competitive phase of action was obtained in the other agonists studied here. Furthermore, Nickerson (1956) reported that the blockade remained virtually unchanged for up to 8 h even though a shift of two log units was obtained. Evidence concerning the wash out of ^{14}C-dibenamine, suggests that more than 75% of loosely bound material is released in 60 min, and the quantity of dibenamine remaining bound to the tissue, in this case vascular smooth muscle, is essentially unchanged after 120 min (Yong & Marks, 1967).

A further hypothesis to explain the different results obtained with phenoxybenzamine and GD-121 could be developed by supposing that the histamine receptors are of two types which interact in the same fashion with the agonist, but differ in their sensitivities to different β-haloalkylamines. The presence of a receptor reserve in such a heterogeneous population of receptors could give rise to the results obtained. While this suggestion is not implausible, it is rather less appealing than another suggestion, mentioned earlier, which was put forward by Moran & Triggle (1970). It would seem probable from the uptake curve for ^{14}C-phenoxybenzamine, and the correlation of this with blockade (Fig. 10) that phenoxybenzamine effects blockade by progressive attack on any nucleophilic groups, rather than by successive inactivation of various receptor systems. The facility with which β-haloalkylamines bind to non-specific sites (Terner, Cook & Marks, 1971) lends further support to this view. It is thus possible that at low concentrations phenoxybenzamine and GD-121 interact with a site which is in the neighbourhood of the receptor and, possibly by causing a conformational change, limit the access of agonist to the receptor without rendering it non-functional. This would produce the observed shift in the dose-response curve. At higher concentrations of antagonist it is supposed that receptor itself would be inactivated, thus leading to a decrease in the maximum response which can be obtained. The relative ease of alkylation of these two sites will determine the nature of the dose response relationship; GD-121 would act at the neighbouring sites, and only when a substantial portion of the sites have interacted would the receptor itself be inactivated. Phenoxybenzamine, on the other hand, would interact with a limited proportion of neighbouring sites before the receptor itself was subject to inactivation, thus effecting a very small shift in dose-response curve.

The absence of a parallel shift in the dose-response curve for acetylcholine does not require any special comment but for the observation that an apparent receptor reserve is very frequently encountered for this agonist. Burgen (1965) showed a small receptor reserve (77%) for acetylcholine, using the guinea-pig ileum preparation and dibenamine as the antagonist. Again this may be explained on the hypothesis discussed above; while the antagonists which produce a parallel shift interact with the neighbouring site, which, according to Moran & Triggle (1970), is involved in binding partial agonists, phenoxybenzamine interacts first with the receptor thus producing depression of the maximum response as soon as any blockade becomes apparent.

While acetylcholine and histamine are thought to interact directly with their receptors to elicit the contractor response, the other agents tested have a more

complex mechanism of action. Using the longitudinal muscle preparation of guinea-pig ileum, Paton & Zar (1965) showed that in this denervated preparation both potassium, at low to moderate concentrations, and 5-HT showed a reduced ability to elicit a contractile response relative to the intact tissue. This suggests that, at any rate, a major part of the action of these agents arises from their ability to cause release of acetylcholine from nerve terminals. Further support for this hypothesis was provided by Gershon (1967), who inhibited acetylcholine release with tetrodotoxin, and found that the responses to potassium and 5-HT were substantially reduced while those to histamine and exogenous acetylcholine were unchanged. The same conclusion was reached by Henderson, Ariëns & Simonis (1968). If phenoxybenzamine is inhibiting the contractile response to these agents by blocking the ability of released acetylcholine to interact with its receptors, it would be supposed that the reduction in response to these agents, achieved with phenoxybenzamine, would be similar to that encountered for exogenous acetylcholine. This is clearly the case in the potassium induced contractions, as may be seen from Fig. 10. It is interesting to note that at high concentrations of antagonist, the potassium response is more resistant to blockade than the response to acetylcholine. This observation agrees with the finding of Gershon, that a portion of the potassium induced contraction is resistant to tetrodotoxin and corresponds to a direct action of potassium on smooth muscle. This direct action would appear to be poorly antagonized by phenoxybenzamine at the concentration used here, but evidence suggests that the direct action may be sensitive to β-haloalkylamines at very high concentrations or long exposure periods (Bevan, Osher & Su, 1962; Shibata & Carrier, 1967; Shibata, Carrier & Frankenheim, 1968).

In contrast to the potassium-induced contractions, those produced by 5-HT are very sensitive to phenoxybenzamine, which completely blocks the responses to this agent at concentrations above 5×10^{-8} g/ml, and reduces the response by 50% at a concentration of 10^{-8} g/ml. The acetylcholine responses are still maintained under these circumstances, and it is thus reasonable to suppose that the antagonism of the 5-HT induced contractions lies not in the antagonism of released transmitter, but in an inhibition of the ability of 5-HT to cause neuronal release of acetylcholine.

Thus of the hypotheses advanced to explain the parallel shift of dose-response curve obtained, in some cases, after irreversible antagonism, the 'two-site' hypothesis appears, on the basis of studies reported here, to be more probable than the 'spare-receptor' hypothesis. Blockade of various agents by phenoxybenzamine may result from (a) interaction at a site which interferes with the receptor, but does not inactivate it, giving rise to an antagonism which appears competitive in nature; (b) direct inactivation of the receptor; (c) inactivation of the receptor for the transmitter released by the agent in question, or (d) prevention of the transmitter releasing activity at the level of the nerve terminal.

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