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Mechanism of potentiation by amines of non-equilibrium blockade of the α-adrenoceptor

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Summary

1. The mechanism by which sympathomimetic and certain other amines enhance blockade of the α-adrenoceptors by the non-equilibrium antagonist *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in strips of rabbit aorta was examined.

2. Non-equilibrium blockade of the 5-hydroxytryptamine receptors by EEDQ was not increased by sympathomimetic amines and was decreased by 5-hydroxytryptamine.

3. Low concentrations of reversible competitive antagonists appeared to protect selectively against the additional blockade by EEDQ which develops in the presence of an amine.

4. Phenoxybenzamine potentiated EEDQ blockade of the α-receptors but not of the 5-hydroxytryptamine receptors.

5. Augmentation of EEDQ blockade was also detected in a variety of other tissues, but not in segments of rabbit intestine where α-adrenoceptors mediate an inhibitory response.

6. It was concluded that EEDQ acts at two sites in antagonizing α-receptor mediated responses, and that one of these sites (site II) is separate from the site of action of agonists and phenoxybenzamine (site I). Amines which enhance blockade appear to exert their action by combining with a third site (site III), which may induce a conformational alteration at site II.

7. It appears that the α-adrenoceptor may have multiple sites for drug interaction.

Introduction

N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), a compound structurally unrelated to the haloalkylamines, is reported to produce a non-equilibrium antagonism of the α-receptors for sympathomimetic amines in smooth muscle (Belleau, Martel, Lacasse, Menard, Weinberg & Perron, 1968; Belleau, DiTullio & Godwin, 1969). In support of the proposed action of EEDQ it was found in experiments on strips of rabbit aorta that EEDQ shifted the dose-response curves to phenylephrine and noradrenaline to the right and decreased their slopes and maximal amplitudes (Kalsner, 1970; 1971). The blockade produced was not reversed significantly after 4 h with frequent washing of the muscle chamber. EEDQ was also active as a 5-hydroxytryptamine antagonist but it had no apparent activity against responses to histamine or potassium.

A further indication of a similarity in the actions of EEDQ and the haloalkylamines was the finding that reversible competitive antagonists appeared to protect equally well against irreversible blockade by EEDQ or phenoxybenzamine. However, in contrast to expectations based on studies with phenoxybenzamine and dibenamine, the blockade of α-adrenoceptor mediated responses by EEDQ was increased, rather than decreased, when strips were exposed to the antagonist in the presence of a sympathomimetic amine. On the basis of this and other evidence it was proposed that certain amines increase the effectiveness of the EEDQ-α-receptor interaction by combining with a stereospecific binding site which is distinct from the α-receptor site, to induce a conformational alteration at the site of EEDQ blockade (Kalsner, 1970 ; 1971).

The present study is a further investigation into the sites of action of EEDQ and the mechanism of potentiation of EEDQ blockade by amines. The results obtained suggest that the α-receptor is not a single site but rather a complex of interacting sites.

Methods

Helically cut strips of thoracic aorta from rabbits weighing between 2 and 4 kg were prepared for isotonic recording as described previously (Kalsner & Nickerson, 1968). The strips were suspended under a tension of 2 g in 15 ml 'drain-out' chambers maintained at 37° C and containing a modified Krebs–Henseleit (Krebs) solution (NaCl, 115·3 mM ; KCl, 4·6 mM ; $CaCl_2$, 2·3 mM ; $MgSO_4$, 1·1 mM ; $NaHCO_3$, 22·1 ; KH_2PO_4, 1·1 mM ; glucose, 7·8 mM) to which the disodium salt of ethylene diamine tetra-acetic acid was added (0·03 mM) to prevent oxidation of catecholamines catalyzed by heavy metals. The chambers were constantly bubbled with 95% O_2–5% CO_2, and the pH of the bathing medium was 7·4. Strips were allowed to equilibrate in the muscle chambers for 90 min prior to drug testing.

The procedure for the preparation of strips of rat and guinea-pig thoracic aorta was the same as described above for the rabbit except that tension on the strips was reduced to 1 g. Rabbit stomach (fundal) strips of about 23 × 2·5 mm and segments of ileum, about 2·5 cm long, were taken from rabbits starved overnight. The preparations were suspended under 2 g tension and a period of equilibration of at least 30 min was allowed before drug testing. The central artery of the rabbit ear was prepared according to the method described by De La Lande, Frewin & Waterson (1967). The left and right ear arteries were cannulated at both ends and mounted in individual 30 ml muscle chambers containing Krebs solution. The vessels were perfused through their lumens with oxygenated Krebs solution at 37° C by means of a gravity-feed apparatus maintained at a pressure of 85 cm of water. The rate of flow of fluid through the vessels was recorded with a modified Gaddum outflow recorder (Andrews, 1952) attached to a piston recorder writing on a kymograph drum.

All drug concentrations are expressed in terms of molarity and also as w/v (g/ml), (−)-adrenaline bitartrate, (−)-phenylephrine hydrochloride, 5-hydroxytryptamine creatine sulphate and histamine hydrochloride in terms of the base, phentolamine hydrochloride and phenoxybenzamine hydrochloride in terms of the salt. *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was dissolved by shaking in propylene glycol to give a stock concentration of 1 or 5 mg/ml (4 or 20 mM), and was always prepared freshly on the day of use.

Results

Effects of exposure of rabbit aortic strips to EEDQ in the presence of amines on blockade of α- and 5-hydroxytryptamine receptors

The records obtained in a typical experiment illustrating the enhancing effect of sympathomimetic amines on EEDQ blockade of the α-adrenoceptors are presented in Figure 1. Adrenaline was effective in potentiating EEDQ blockade over a wide concentration range (10 ng/ml–30 μg/ml; 55 nM–0·16 mM), and phenylephrine and noradrenaline had a similar effect. In contrast, sympathomimetic amines decreased blockade by phenoxybenzamine, presumably reflecting competition for occupancy of receptor sites (Fig. 2).

In aortic strips from some preparations the peak response to a given concentration of agonist was fading when the next higher concentration was added during the cumulative concentration-effect curves. The tendency for the contraction to fade from an initial peak amplitude to a slightly lower plateau level was observed occasionally but was unrelated to the experimental conditions. This was confirmed by comparing fade in control strips, strips treated with EEDQ alone and with EEDQ in the presence of an amine. When equal responses to phenylephrine were produced in all strips so as to ensure equivalent degrees of receptor activation, no consistent differences in the degree of fade were observed.

In a previously reported study of structure-activity relationships, it was observed that a compound need not have sympathomimetic or even smooth muscle stimulat-

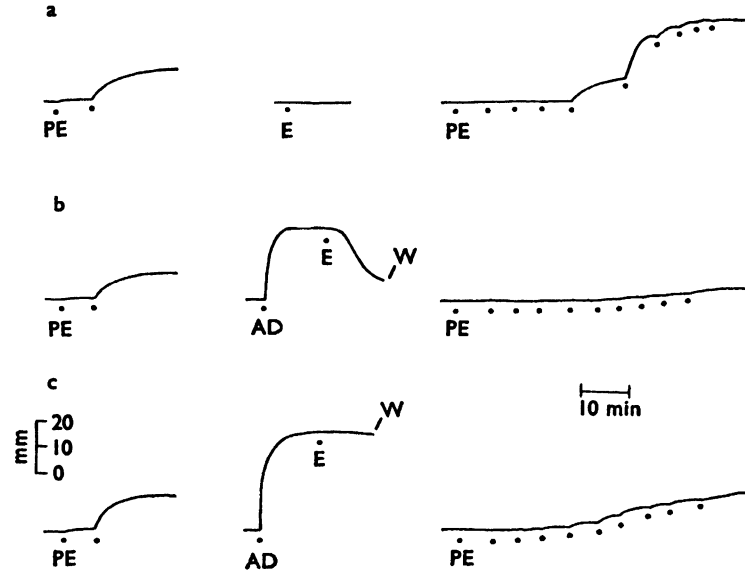

FIG. 1. Blockade of α-adrenoceptors in rabbit aortic strips by EEDQ in the presence and absence of adrenaline. Left: (a), (b) and (c), test responses of 3 strips from the same aorta to cumulative concentrations of phenylephrine (PE) (3, 10 ng/ml; 18, 60 nM) (dots). Centre: (a) strip exposed to EEDQ (E) (2 μg/ml; 8·1 μM) alone for 10 min and then washed; (b) strip contracted by adrenaline (AD) (60 ng/ml; 330 nM) followed after 10 min by EEDQ and washed (W) 10 min later; (c) same as in (b) but adrenaline concentration increased to 16 μM. Right: (a), (b) and (c), responses of each strip to phenylephrine (3, 10 and 30 ng/ml, 0·1 and 0·3, 1 and 3, 10 and 30, 100 μg/ml; 18 nM to 0·6 mM) 60 min after washout of EEDQ. Responses of control strips to phenylephrine (18 and 60 nM) were unchanged during the course of the experiment, the position of a typical control dose-response curve relative to that obtained after treatment with EEDQ is shown in Figure 4.

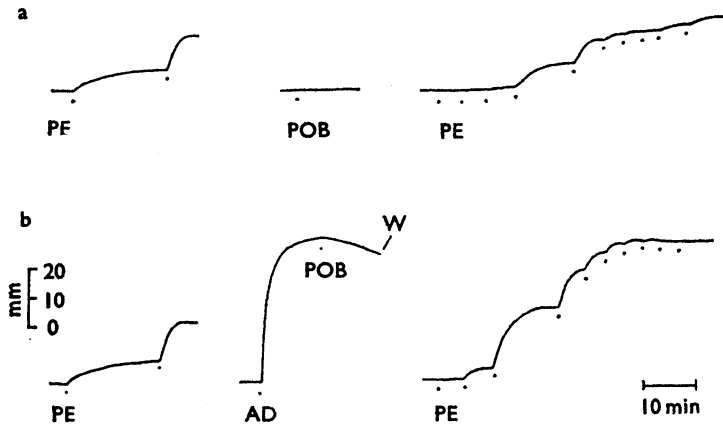

FIG. 2. Blockade of the α-adrenoceptors in rabbit aortic strips by phenoxybenzamine in the presence and absence of adrenaline. Left: (a) and (b) test responses of 2 strips from the same aorta to cumulative concentrations of phenylephrine (PE) (3, 10 ng/ml; 18, 60 nM) (dots). Centre: (a) strip exposed to phenoxybenzamine (POB) (10 ng/ml; 30 nM) alone for 10 min and then washed; (b) strip contracted by adrenaline (AD) (3 μg/ml; 18 μM) followed after 10 min by POB and washed (W) 10 min later. Right: (a) and (b), responses of each strip to phenylephrine (3, 10 and 30 ng/ml, 0·1 and 0·3, 1 and 3, 10 and 30, 100 μg/ml; 18 nM to 0·6 mM) 60 min after washout of POB.

ing activity to enhance EEDQ blockade (Kalsner, 1970). Although the most active compounds had the phenethylamine nucleus the essential requirement for activity appeared to be an aliphatic chain with a primary or secondary amine group. Out of a total of about forty compounds tested the most potent, in terms of the molar concentration required to enhance blockade, were adrenaline> phenylephrine>5-hydroxytryptamine>noradrenaline.

EEDQ is about equally effective in blocking α-adrenoceptors and 5-hydroxytryptamine receptors (Kalsner, 1970). However, as was previously reported, sympathomimetic amines in concentrations up to 10 μg/ml did not affect in any way blockade of the 5-hydroxytryptamine receptors by EEDQ. Experiments were done, in which the technique of receptor protection was used (Furchgott, 1954), to determine if 5-hydroxytryptamine would protect its own receptors against blockade by EEDQ. Aortic strips were exposed to EEDQ (4 μg/ml; 16 μM) in the presence and absence of 5-hydroxytryptamine (3 μg/ml; 17 μM) and the degree of blockade was determined 90 min later with cumulatively increasing concentrations of 5-hydroxytryptamine (Fig. 3). It is evident from Fig. 3 that the agonist significantly protected its own receptors against EEDQ blockade.

The effect of phentolamine on enhancement of EEDQ blockade

Reversible competitive antagonists of the α-adrenoceptors, such as phentolamine, nylidrin and chlorpromazine protected about equally well against irreversible blockade by EEDQ or phenoxybenzamine (Kalsner, 1970), suggesting that all these compounds compete for a common site. However, in the present experiments it was observed that low concentrations of competitive reversible antagonists were considerably more effective in protecting against the additional component of EEDQ block which develops in the presence of an amine.

The results of a typical experiment are presented in Figure 4. The control dose-response curve to phenylephrine was shifted to the right and the maximal response

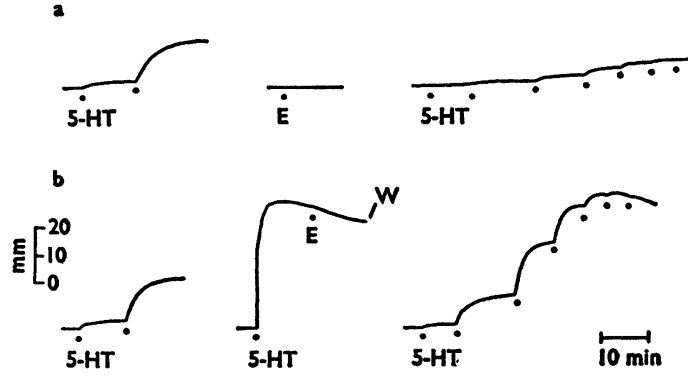

FIG. 3. Blockade of the 5-hydroxytryptamine receptors by EEDQ in the presence and absence of 5-hydroxytryptamine. Left: (a) and (b) test responses of 2 strips from the same rabbit aorta to 5-hydroxytryptamine (5-HT) (3, 10 ng/ml; 17, 56 nM) (dots). Centre: (a) strip exposed to EEDQ (E) (4 μg/ml; 16 μM) alone for 10 min and washed; (b) strip contracted by 5-hydroxytryptamine (3 μg/ml; 17 μM) followed after 10 min by EEDQ and washed (W) 10 min later. Right: (a) and (b) responses of each strip to 5-HT (3, 10 and 30 ng/ml, 0·1 and 0·3, 1 and 3 μg/ml; 17 nM to 17 μM) 90 min after washout of EEDQ.

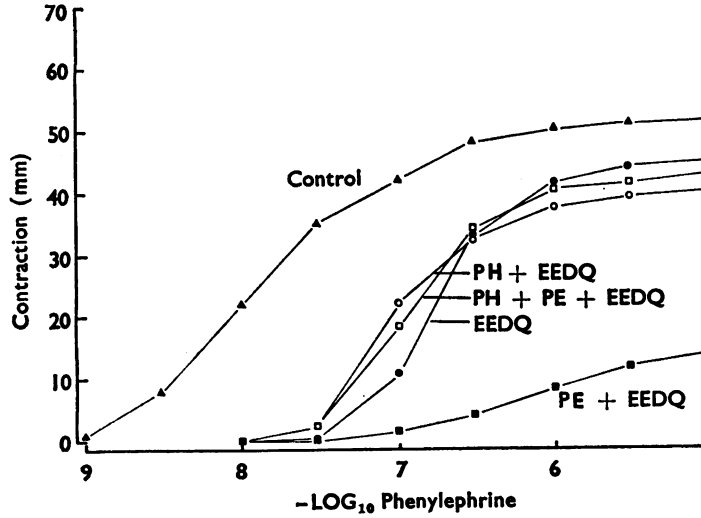

FIG. 4. Phentolamine inhibition of enhancement of EEDQ blockade. All 5 strips were taken from the same rabbit aorta and tested initially with phenylephrine (3, 10 ng/ml; 18, 60 nM) to establish matching sensitivities. Reading from left to right on the graph the strips were treated as follows: (1) control; (2) exposed to phentolamine (PH) and 20 min later, without washout, to EEDQ for an additional 10 min; (3) exposed to phentolamine and 10 min later, without washout to phenylephrine (PE) (10 μg/ml; 60 μM) followed after 10 min by EEDQ for 10 min; (4) exposed to EEDQ for 10 min; (5) exposed to phenylephrine (60 μM) followed after 10 min by EEDQ for 10 minutes. The concentrations of EEDQ and phentolamine were 2 μg/ml (8·1 μM) and 20 ng/ml (63 nM) respectively. Sixty min after washout of the antagonist all strips were contracted simultaneously by cumulatively increasing concentrations of phenylephrine (1 ng/ml to 10 μg/ml).

height decreased by a 10 min exposure to EEDQ (2 μg/ml; 8·1 μM). In the presence of phenylephrine (10 μg/ml; 60 μM) the blockade was increased considerably. As can be seen (Fig. 4), the blockade by EEDQ alone may be slightly decreased in the presence of a low concentration of phentolamine (20 ng/ml; 63 nM), but the enhancement of blockade by phenylephrine (10 μg/ml; 60 μM) is completely eliminated. Phentolamine similarly inhibited the block-enhancing action

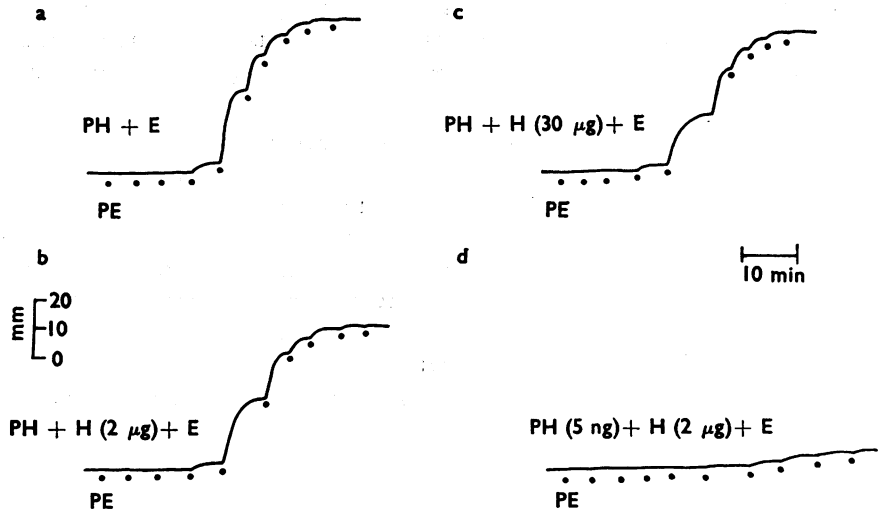

FIG. 5. Effects of changes in concentration of enhancing amine or of phentolamine on phentolamine inhibition of enhancement of EEDQ blockade. All rabbit aortic strips were matched as regards initial sensitivity to phenylephrine. (a) Strip exposed to phentolamine (PH) (50 ng/ml; 158 nM) for 20 min and without washout, to EEDQ (E) (2 μg/ml; 8·1 μM) for an additional 10 min; (b) strip exposed to phentolamine and 10 min later, without washout, to histamine (2 μg/ml; 18 μM) followed after 10 min by EEDQ for 10 min; (c) same as in (b) but histamine concentration increased to 30 μg/ml (270 μM); (d) same as in (b) but phentolamine concentration decreased to 5 ng/ml (16 nM). The contractions shown are to cumulatively increasing concentrations of phenylephrine (PE) (1 and 3, 10 and 30 ng/ml, 0·1 and 0·3, 1 and 3, 10 and 30 μg/ml; 6 nM to 180 μM) 60 min after washout of the antagonist.

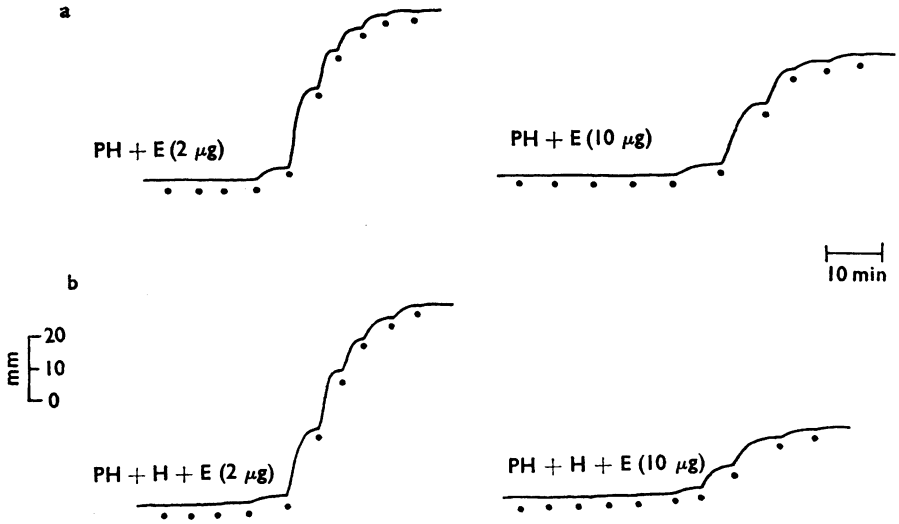

FIG. 6. Effect of changes in concentration of EEDQ on phentolamine protection against enhancement of EEDQ blockade. All strips were taken from the same rabbit aorta and matched as regards initial sensitivity to phenylephrine. (a) Strips exposed to phentolamine (PH) (50 ng/ml; 158 nM) for 20 min and, without washout, to EEDQ (E) (2 or 10 μg/ml; 8·1 or 40 μM) for 10 min; (b) strips exposed to phentolamine for 10 min and, without washout, to histamine (H) (2 μg/ml; 18 μM) for 10 min followed by EEDQ (2 or 10 μg/ml; 8·1 or 40 μM). The contractions shown are to cumulatively increasing concentrations of phenylephrine (PE) (1 ng/ml to 30 μg/ml; 6 nM to 180 μM) 90 min after washout of the antagonist.

of tyramine (10 μg/ml ; 73 μM) and histamine (3–30 μg/ml ; 27–270 μM). Results similar to those shown in Fig. 4 were obtained in a total of 14 experiments. In addition, the same effect was obtained when nylidrin (0·1 μg/ml ; 0·3 μM) was used instead of phentolamine.

The relatively selective inhibition by a low concentration of phentolamine of the enhancement of EEDQ blockade could be due either to competition between the phentolamine and EEDQ or between phentolamine and the enhancing amine for a specific receptor site. These possibilities were explored by attempting to break through the inhibition by increasing the concentration of amine or of EEDQ. Figure 5 shows an experiment in which the phentolamine concentration (50 ng/ml ; 158 nM) was not greatly in excess of that needed to inhibit completely the enhancing effect of 18 μM histamine (compare panels b and d). A 15-fold increase in the histamine concentration (panel c) nonetheless failed to overcome the effect of the

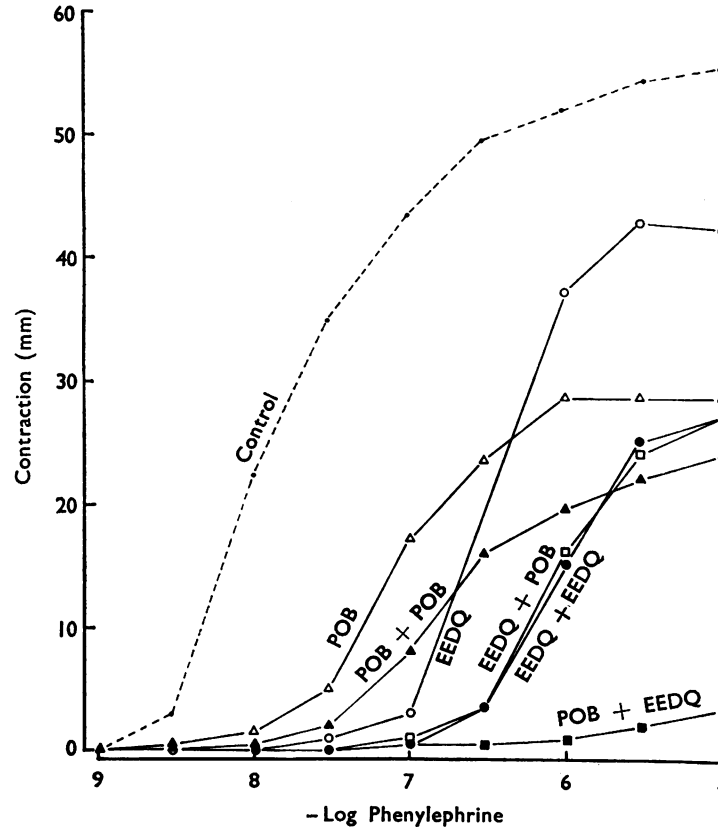

FIG. 7. Potentiation of EEDQ blockade by phenoxybenzamine (POB). All strips were taken from the same rabbit aorta and tested initially with phenylephrine (3, 10 ng/ml ; 18, 60 nM) to establish matching sensitivities. Reading from left to right on the graph the strips were treated as follows: (1) control ; (2) exposed to POB for 10 min ; (3) exposed to POB for 10 min and after a 30 min interval re-exposed to POB ; (4) exposed to EEDQ for 10 min ; (5) exposed to EEDQ for 10 min and 30 min later to POB for 10 min ; (6) exposed to EEDQ for 10 min and after a 30 min interval re-exposed to EEDQ ; (7) exposed to POB and after a 30 min interval to EEDQ. POB and EEDQ were used in concentrations of 5 ng/ml (15 nM) and 2 μg/ml (8·1 μM) respectively. All strips were contracted simultaneously by cumulative concentrations of phenylephrine (1 ng/ml to 10 μg/ml) 60 min after washout from the second exposure to an antagonist.

phentolamine. It was similarly found that increasing the concentration of phenylephrine did not restore the enhancing effect in the presence of a low concentration of phentolamine suggesting that the interaction between phentolamine and the enhancing amines is not competitive in nature. However, raising the concentration of EEDQ did lead to the reappearance of enhanced blockade (Fig. 6). This was confirmed in 5 experiments. Both the magnitude of sub-maximal responses and the maximal response amplitude were significantly decreased by the enhancing amine in the presence of phentolamine (50 ng/ml; 158 nM) and EEDQ (10 μg/ml; 40 μM).

Potentiation of EEDQ blockade by phenoxybenzamine

Aortic strips were exposed twice to phenoxybenzamine (5 ng/ml; 15 nM) or twice to EEDQ (2 μg/ml; 8·1 μM), or successively to EEDQ and phenoxybenzamine in either sequence, and the total blockade produced was assessed 60 min later with phenylephrine as test agonist (Fig. 7).

Two exposures to phenoxybenzamine produced somewhat greater blockade than a single exposure and the same was found with EEDQ. The blockade produced by EEDQ followed by phenoxybenzamine was slightly greater than the blockade by EEDQ alone, but the sequence of phenoxybenzamine followed by EEDQ virtually eliminated responses to phenylephrine. These results were confirmed in a total of 20 tests. This effect appeared to be specific for α-receptors, and was not seen when the agonist was 5-hydroxytryptamine (Fig. 8).

Attempts were made to protect against phenoxybenzamine enhancement of EEDQ blockade of the α-adrenoceptors by incubating strips with the haloalkylamine in the presence of an enhancing amine. It was found that high concentra-

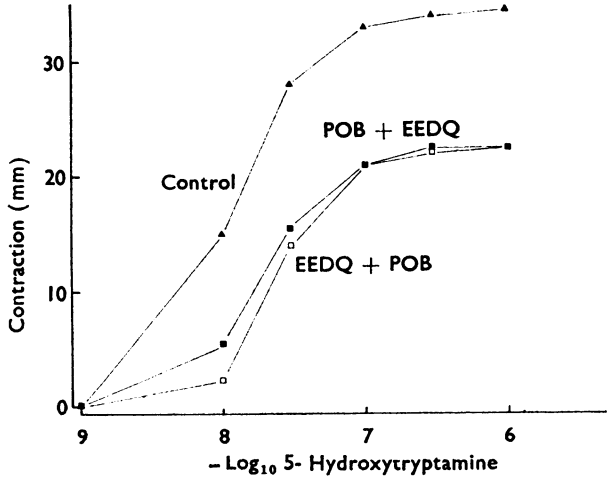

FIG. 8. Lack of potentiation of EEDQ block of 5-hydroxytryptamine receptors by phenoxybenzamine (POB). All strips were taken from the same rabbit aorta and tested initially with 5-hydroxytryptamine (10, 30 ng/ml; 56, 168 nM) to establish matching sensitivities. Reading from left to right on the graph the strips were treated as follows: (1) control; (2) exposed to POB (40 ng/ml; 120 nM) for 10 min and 30 min later to EEDQ (2 μg/ml; 8·1 μM) for 10 min; (3) exposed to EEDQ (8·1 μM) for 10 min and 30 min later to POB (120 nM), for 10 minutes. All strips were contracted simultaneously by cumulative concentrations of 5-hydroxytryptamine (1 ng/ml to 1 μg/ml) 60 min after washout from the second exposure to an antagonist.

tions of histamine (3 μg/ml ; 27 μM), phenethylamine (3 μg/ml ; 25 μM) or 5-hydroxytryptamine (3 μg/ml ; 17 μM) did not reduce the effectiveness of phenoxybenzamine in enhancing the blockade produced by a subsequent exposure to EEDQ. Similarly, incubation of strips with phenoxybenzamine in the presence of phentolamine (50 ng/ml ; 158 nM) did not diminish the enhancement of EEDQ blockade by the haloalkylamine.

Effects of amines on EEDQ blockade in other smooth muscle preparations

The ability of certain amines to enhance EEDQ blockade of α-adrenoceptor mediated responses was examined in several other smooth muscle preparations obtained from the rabbit, rat and guinea-pig. The results obtained are shown in Table 1. Enhancement of blockade was demonstrable in the perfused central

TABLE 1. *Effect of amines on EEDQ blockade in several smooth muscle preparations*

Preparation	EEDQ conc. in μM	Enhancing amine (μM)	No. strips	Agonist dose-ratio
Rat aorta	12		2	65
	12	5-HT (17)	2	>3000
Guinea pig aorta	12		2	100
	12	5-HT (17)	2	>2000
Rabbit central ear artery	40		5	12
	40	5-HT (17)	2	>2500
Rabbit stomach	12		3	375
	12	5-HT (17)	2	>3000
	12	phenethylamine (25)	1	>3000
Rabbit ileum	12		2	100
	12	5-HT (17)	1	100
	12	phenylephrine (0·6)	1	100
	20		3	700
	20	phenylephrine (18)	4	775
	20	5-HT (17)	1	650

Tissues were prepared as described in **Methods**, and tested initially with low concentrations of agonist to establish sensitivity. They were then exposed to EEDQ alone for 10 min, or first to an enhancing amine for 10 min and then, without washout, to EEDQ for an additional 10 minutes. 30 to 60 (usually 60) min after washout of the antagonist all preparations were contracted by increasing concentrations of agonist. Agonist dose-ratio refers to the ratio of the concentrations of agonist required for an equivalent small response 60 min after and 30 min before exposure to the antagonist. Phenylephrine was the agonist in all cases except in the central artery of the rabbit ear, where noradrenaline was used.

artery of the rabbit ear and in the rat and guinea-pig aorta. However, this effect was not limited to vascular tissue. Responses of rabbit stomach strips to phenylephrine were significantly more depressed after exposure to EEDQ plus phenethylamine or 5-hydroxytryptamine than to EEDQ alone. Exposure of these preparations to the enhancing amine in the absence of the antagonist did not decrease their sensitivity to subsequently elicited responses to the test agonist.

The only preparation tested in which the phenomenon of enhanced blockade could not be detected was the rabbit ileum. Intestinal smooth muscle contains both α- and β-adrenoceptors, both subserving relaxation, and phenylephrine was employed as the test agonist since it has negligible β-receptor activity in this preparation (Furchgott, 1960 ; Kalsner, unpublished). EEDQ appeared to produced a characteristic non-equilibrium blockade, but it was not increased in the presence of 5-hydroxytryptamine or phenylephrine.

Discussion

The blockade of the α-adrenoceptors produced by EEDQ is in some ways similar to that observed with the haloalkylamines. However, a striking dissimilarity appears when aortic strips are incubated with the antagonists in the presence of a sympathomimetic amine. Whereas compounds such as adrenaline, noradrenaline and phenylephrine provide protection against phenoxybenzamine blockade they enhance EEDQ blockade. A previous structure-activity study (Kalsner, 1970) revealed that although the requirements for α-receptor activation and enhancement of blockade are fairly similar, there are sufficient differences to indicate that this latter action of amines is exerted at a site which is different from the site through which agonists initiate a response.

For example, 5-hydroxytryptamine and phenethylamine (the latter compound after inhibition of monoamine oxidase to prevent its rapid deamination) are both about as potent as adrenaline, and at least twice as potent as noradrenaline in augmenting blockade. These compounds are neither α-receptor stimulants nor antagonists at the concentrations employed. The finding that the (−)-isomer of amphetamine is about 10 times as potent as the (+)-isomer, although the order of potency in contracting smooth muscle is reversed, served further to indicate that amines react with a specific tissue site to enhance blockade (Kalsner, 1970). The specificity of the enhancing effect for the α-adrenoceptors was confirmed by the observation that non-equilibrium blockade of the 5-hydroxytryptamine receptors by EEDQ was not increased by amines.

The results obtained in the present investigation suggest that EEDQ acts at more than one site to block the effects of sympathomimetic amines and this may provide information as to why amines augment blockade by EEDQ but not by phenoxybenzamine. The failure of sympathomimetic amines to protect the α-receptors against EEDQ although 5-hydroxytryptamine markedly decreased the level of blockade of its own receptors, suggests that EEDQ and 5-hydroxytryptamine act at the same site, but that at least a part of the action of EEDQ in blocking α-adrenoceptor mediated responses is exerted at a site other than that with which agonist drugs combine.

The possibility of two sites of action of EEDQ was supported by the finding that phentolamine protected completely against the component of EEDQ block which develops in the presence of an amine, at a concentration which gave only slight protection against blockade by EEDQ alone. Evidence that this involved protection of a tissue site against occupancy by EEDQ was the observation that enhancement could be restored by raising the concentration of EEDQ but not by an increase in the level of the enhancing amine. Although no evidence was obtained to support the view that phentolamine protects against enhancement by occupying the site with which amines react, this alternative possibility should not be dismissed.

The experiments in which phenoxybenzamine and EEDQ were administered in sequence provided additional information on the sites of action of EEDQ. Phenoxybenzamine potentiated EEDQ blockade of the α-receptors but not the 5-hydroxytryptamine receptors. This again points to the conclusion that the two α-receptor antagonists block, at least in part, at different sites. The augmentation of EEDQ blockade by phenoxybenzamine, and by amines, appears to involve different mechanisms. It was found that exposure to phenoxybenzamine in the

presence of a high concentration of a non-sympathomimetic amine with enhancing action or in the presence of phentolamine (50 ng/ml ; 158 nM), did not protect in any way against phenoxybenzamine potentiation of EEDQ blockade.

It appears that one of the two sites at which EEDQ acts to block α-adrenoceptor mediated responses is the traditionally accepted site of action of phenoxybenzamine and of agonists in blocking or initiating a response (site I). EEDQ appears to act at site I when it is given alone but the extent of its action at this site is not clear. Reversible competitive antagonists protected about equally well against blockade by EEDQ given alone or phenoxybenzamine, and so did the short-acting non-equilibrium haloalkylamine antagonist *N,N*-dimethyl 2-chloro phenethylamine, a compound with a chemical reactivity similar to that of phenoxybenzamine (Kalsner, 1970). Sympathomimetic amines were also able to protect to some extent against EEDQ blockade, although this was generally masked by their dominant enhancing effect. For example, by comparing the effects of a high and of a low concentration of adrenaline on EEDQ blockade (Fig. 1), it can be seen that a greater potentiation results from the lower concentration. This was not observed with amines lacking sympathomimetic properties.

The second site of EEDQ action (site II) appears to be the site at which the EEDQ-receptor interaction is enhanced by amines and is probably also a site of action of EEDQ given alone. As was indicated above, phentolamine effectively inhibits enhancement of block by amines by protecting against EEDQ occupancy at site II. It is proposed that amines in enhancing blockade act at a third site (site III) and induce a conformational alteration at site II. Since phenoxybenzamine does not act to any significant extent at site II its blocking effects are not enhanced by amines. Phenoxybenzamine by occupying site I may also alter the conformation of site II, thus explaining its augmentation of EEDQ blockade.

Other evidence that phenoxybenzamine and EEDQ have different modes of action is obtained from an examination of dose-response curves after blockade by these agents (Fig. 7). Phenoxybenzamine produces a greater reduction in maximal response amplitude for a given shift to the right of the dose-response curve than does EEDQ. This may reflect differences in the sites of action of these compounds.

The mechanism of irreversible blockade by EEDQ is not known with certainty but Belleau *et al.* (1969) have accumulated considerable evidence to indicate that blockade proceeds via formation of a mixed acid anhydride intermediate involving a receptor carboxyl group. This is followed by the acylation of a nucleophile on the receptor surface by the carbonyl function of the receptor carboxyl group, leading to cross-linkage formation and α-receptor inactivation.

The possibility of drug-induced conformation changes in receptor structure has been considered by others. For example, Rang & Ritter (1969) invoked a conformational alteration to explain the enhancement by some agonists of the blocking activity of certain curare-like agents on the chick biventer cervicis and leech muscle. They proposed that stimulation by agonists led to a structural change in the receptor. However, the present experiments demonstrate that augmented blockade by EEDQ involves occupancy of a site which is distinct from the site involved in stimulation. Another example of altered receptor affinity for antagonist molecules was noted by Karlin & Winnick (1968). These workers found that *N*-ethyl-maleimide inactivated the acetylcholine receptors of the electroplax only after a disulphide bond on the receptor surface was first reduced by dithiothreitol. The

possibility of multiple sites of drug interaction on receptor surfaces has also been considered (Changeux, Thiery, Tung & Kittel, 1967; Changeux, Blumenthal, Kasai & Podleski, 1970). These workers discussed the implications of allosteric regulation of receptor function. It may be that the sites of interaction of amines and antagonists observed in the present experiments are related to as yet unidentifiable allosteric transitions.

The physiological implications of the present proposal that the α-adrenoceptor is a complex of sites, rather than a single site, are not clear. The phenomenon of enhanced blockade was detectable not only in rabbit aorta but also in several other smooth muscle preparations from different species. Enhancement could not be detected in rabbit intestine. Whether this is related to the fact that α-receptors mediate an inhibitory response in intestine and are therefore structurally different from those examined in other preparations, cannot be answered by the present experiments.

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