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Molecular Pharmacology of *Alpha* Adrenergic Receptors: Utilization of [^{3}H]Dihydroergocryptine Binding in the Study of Pharmacological Receptor Alterations

LEWIS T. WILLIAMS[1] AND ROBERT J. LEFKOWITZ[2]

Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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SUMMARY

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The potent *alpha* adrenergic antagonist [^{3}H]dihydroergocryptine has been shown to bind reversibly to *alpha* adrenergic receptors in uterine smooth muscle membranes. [^{3}H]Dihydroergocryptine binding is antagonized by phentolamine and phenoxybenzamine. Inhibition of [^{3}H]dihydroergocryptine binding by phentolamine, a reversible inhibitor of adrenergic smooth muscle contraction, was reversible and resulted in a reduction in the apparent affinity of *alpha* adrenergic receptors with no change in the number of available receptor binding sites. By contrast, phenoxybenzamine, an irreversible inactivator of adrenergic smooth muscle contraction, irreversibly inactivated *alpha* adrenergic receptor binding sites as assessed by [^{3}H]dihydroergocryptine binding. Irreversible receptor inactivation by phenoxybenzamine occurred rapidly ($t_{1/2} \sim 1$ min), even at low drug concentrations (0.1 μM). Exposure of membranes to 0.1 μM phenoxybenzamine resulted in a 50% reduction in the number of receptor sites, with little or no change in the apparent affinity of the unreacted binding sites for [^{3}H]dihydroergocryptine. This characteristic nonequilibrium blockade of *alpha* adrenergic receptors contrasts with the competitive blockade produced by phentolamine and ergotamine. The specific sulfhydryl inhibitor *p*-hydroxymercuribenzoic acid inhibited [^{3}H]dihydroergocryptine binding, causing half-maximal inhibition at a concentration of 0.3 mM. This inhibition of binding was reversed or prevented by 2 mM dithiothreitol, a sulfhydryl-containing compound. Thus the *alpha* adrenergic receptor binding site appears to possess an essential sulfhydryl group. The presence or absence of calcium did not alter [^{3}H]dihydroergocryptine binding and did not alter the affinity of epinephrine for the binding sites. These results indicate that, unlike competitive antagonists, haloalkylamine *alpha* adrenergic antagonists act directly to inactivate the *alpha* adrenergic receptor binding sites irreversibly by covalent bond formation. The mechanism of this inactivation may involve the alkylation of an essential sulfhydryl group at the *alpha* adrenergic binding site. In contrast, the effect of calcium ion on *alpha* adrenergic responses is not at the receptor binding site but at some distal step.

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[2] Established Investigator of the Howard Hughes Medical Institute.

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INTRODUCTION

Catecholamine-induced smooth muscle contraction is an *alpha* adrenergic response (1). Adrenergic agonists elicit this response in a specific order of potency (epinephrine ≧ norepinephrine ≫ isoproterenol), and a variety of competitive antagonists (e.g., phentolamine) specifically block the response. Presumably, *alpha* adrenergic smooth muscle contraction is initiated by the reversible interaction of agonist drugs or hormones with specific *alpha* adrenergic receptor sites located in the smooth muscle cells of the responding tissue. Activation of the receptors leads to a sequence of as yet unidentified steps which ultimately result in smooth muscle contraction.

During the last 20 years a large number of experiments *in vitro* have been performed in attempts to gain insight into the nature of the interaction between *alpha* adrenergic agonists and their specific *alpha* adrenergic receptors. Until very recently studies of the interaction of drugs with *alpha* adrenergic receptors have been limited by the necessity of inferring information about the receptor from measurements of the contractile response, which is the final step in the sequence initiated by the drug-receptor interaction. Based on these early experiments and on a classical pharmacological theory, there are several distinct types of mechanisms by which pharmacological interventions might alter *alpha* adrenergic responses to agonist drugs.

1. Competitive antagonists (such as phentolamine) reversibly occupy the receptor sites and block the response to agonists.

2. Irreversible antagonists (such as haloalkylamines or sulfhydryl-reactive reagents) chemically alter some essential cellular component involved in the reaction sequence of the *alpha* adrenergic response to catecholamines. It has been suggested that the irreversible inactivation of *alpha* adrenergic responses by haloalkylamines is due to a direct alteration of the *alpha* adrenergic binding site (2). However, because of the existence of spare receptors (3) and/or nonlinear receptor–occupancy response relationships (4), the extent of inhibition of the contractile response to an *alpha* adrenergic agonist caused by treatment with a β-haloalkylamine does not give a direct measure of the extent of interaction of the β-haloalkylamine with the *alpha* adrenergic binding site. Similarly, the inactivation of *alpha* adrenergic responses by sulfhydryl-reactive reagents, such as PHMB,[3] has been postulated to occur at the level of *alpha* adrenergic receptors (5), although this has never been directly demonstrated and a more distal site of action has not been ruled out.

3. Alterations of components of the cell other than the receptor can affect *alpha* adrenergic responses. For example, alterations in the extracellular concentration of essential cations such as calcium ions can influence the *alpha* adrenergic response to agonists. The sites of such effects are unknown but may involve steps distal to the interaction of agonist with its receptor binding site (6).

The results of the pharmacological studies *in vitro* such as those cited above have left unanswered several uncertainties about the mechanism by which *alpha* adrenergic receptor alterations are produced by haloalkylamines, sulfhydryl reagents, and divalent cations. These uncertainties are the results of attempts to infer information about the molecular characteristics of receptor site interactions from experimental measurements of *alpha* adrenergic responses.

Recently techniques have been described for the direct identification of *alpha* adrenergic receptors by radioligand binding studies (7–9). We have utilized the binding of the *alpha* adrenergic antagonist [^{3}H]dihydroergocryptine to identify *alpha* adrenergic receptor binding sites directly in rabbit uterine smooth muscle membranes (7, 8). In the present studies we have used these new techniques to investigate directly the sites of action of a variety of reagents which alter *alpha* adrenergic responses. These include a reversible competitive *alpha* adrenergic antagonist, irre-

[3] The abbreviations used are: PHMB, *p*-hydroxymercuribenzoate; DTT, dithiothreitol.

versible antagonists such as phenoxybenzamine, group-specific reagents such as sulfhydryl-reactive compounds, and cations such as Ca^{2+}. In addition, the equilibrium and kinetic properties of the interaction of reversible and irreversible antagonists with the *alpha* adrenergic receptor binding sites have been investigated. The results provide new insights into the molecular characteristics of the interactions of these compounds with *alpha* adrenergic receptors.

MATERIALS AND METHODS

Radioligand. [^{3}H]Dihydroergocryptine (specific activity, 23.0 Ci/mmole) was prepared at New England Nuclear by catalytic reduction of ergocryptine (Sigma), using tritium gas, and was purified by thin-layer chromatography. The tritiated compound was chromatographically homogeneous and indistinguishable from authentic dihydroergocryptine (Sandoz) as previously described (8). The compound was stable when stored at −20° in ethanol. No degradation of the compound was detectable during a 17-min incubation with uterine membranes at 25° (8).

Compounds. Other drugs used in this study were (−)-epinephrine bitartrate and ergotamine tartrate (Sigma), phenoxybenzamine (Smith Kline & French), phentolamine hydrochloride (Ciba-Geigy), *p*-hydroxyomercuribenzoate (Sigma), and dithiothreitol (Cyclo).

Membrane preparations. Rabbit uterine smooth muscle membranes were prepared from frozen rabbit uteri (type II, mature) from Pel-Freez Biologicals. Membranes prepared from these uteri gave binding results indistinguishable from those obtained with membranes from fresh New Zealand white rabbit uteri (8). Uteri were used within 3 weeks of freezing. [^{3}H]Dihydroergocryptine binding sites in frozen uteri stored at −70° were stable for up to 6 months. Uteri were cleaned of fat, opened longitudinally, and stripped of endometrium, using a scalpel. Uteri were then minced and homogenized in ice-cold buffer (0.25 M sucrose, 1 mM $MgCl_2$, and 5 mM Tris, pH 7.5) for four 6-sec periods, using a Tekmar tissue grinder at high speed. After filtration through a single layer of gauze, the homogenate was centrifuged at 400 × *g* for 10 min at 4°, and the pellet was discarded. The supernatant was centrifuged at 28,000 × *g* for 10 min at 4°. The resulting pellet was washed in ice-cold incubation buffer (10 mM $MgCl_2$ and 50 mM Tris, pH 7.5) by resuspension and centrifugation at 28,000 × *g* for 10 min. The final pellet was resuspended in incubation buffer at 3–4 mg of protein per milliliter for use in the designated incubations and binding assays.

Preliminary incubations. Preliminary incubations of the membranes with adrenergic antagonists or group-specific reagents were carried out at 25° in a total volume of 1 ml with shaking for the indicated intervals. In the studies with adrenergic antagonists, membranes were washed as follows. The incubation mixture was diluted with 40 ml of 25° incubation buffer and allowed to stand for 10 min at room temperature before centrifugation at 28,000 rpm for 10 min. The pellet was resuspended and similarly washed once more in buffer at 25° and a third time in buffer at 4°. The final pellet was resuspended in incubation buffer for use in the binding assay.

Binding assay. [^{3}H]Dihydroergocryptine (16 nM unless otherwise specified) and uterine membranes (about 4 mg of protein per milliliter unless otherwise specified) were incubated for 15 min (unless otherwise specified) at 25° with shaking in a total volume of 150 μl of incubation buffer. In binding competition experiments the competing agent was added directly to the incubation. Incubations were terminated by diluting 125-μl incubation aliquots with 2 ml of incubation buffer (25°), followed by rapid filtration through Whatman GFC glass fiber filters. Filters were rapidly washed with 20 ml of incubation buffer (25°). Dilution of the membranes and washing of the filters required less than 15 sec. This amount of washing did not reduce specific binding of [^{3}H]dihydroergocryptine but significantly reduced its nonspecific binding (see below). After drying, filters were counted in a Triton-toluene scintillation mixture at an efficiency of 40%.

Nonspecific binding is defined as binding which is not displaced by a high concentration (10 μM) of phentolamine, a potent *alpha* adrenergic antagonist which should occupy essentially all the *alpha* adrenergic receptor binding sites when the standard concentration of 16 nM [³H]-dihydroergocryptine is used. Specific or receptor binding is defined as total radioactivity bound minus nonspecific binding, and was generally 75–90% of the total counts bound to membrane protein. A small amount of [³H]dihydroergocryptine (0.5–0.6% of the total counts filtered) was also nonspecifically adsorbed to the glass fiber filters in the absence of protein. This filter blank was not displaced by phentolamine or other agents. [³H]Dihydroergocryptine binding in the figures refers to specific binding as defined above. Specific [³H]dihydroergocryptine binding was linearly related to the concentration of membrane protein in the assay over a range from 1.0 to 6.0 mg/ml.

Protein was determined by the method of Lowry *et al.* (10).

RESULTS

Reversibility of receptor blockade by adrenergic antagonists. Previous publications from this laboratory have demonstrated that [³H]dihydroergocryptine binds to uterine membranes rapidly and reversibly (7, 8). The binding sites labeled by [³H]dihydroergocryptine have the specificity characteristics expected of *alpha* adrenergic receptors. The *alpha* adrenergic antagonists phentolamine and phenoxybenzamine occupy the binding sites, causing half-maximal inhibition of [³H]-dihydroergocryptine binding at a concentration of about 30 nM (8).

Previous intact tissue studies demonstrated that phentolamine blocks *alpha* adrenergic responses in a reversible, apparently competitive manner (4), whereas phenoxybenzamine (a haloalkylamine) leads to irreversible, nonequilibrium blockade of the *alpha* adrenergic contractile response (2, 3). Using [³H]dihydroergocryptine as a marker for *alpha* adrenergic receptors, we designed experiments to determine whether the relative irreversibility of the phenoxybenzamine-induced blockade of the contractile response is due to an irreversible interaction of the antagonist directly with the receptor sites. In the presence of 1 μM phentolamine or 1 μM phenoxybenzamine, approximately 90% of [³H]dihydroergocryptine binding to uterine membranes was inhibited (Fig. 1A). When membranes previously exposed to 1 μM phentolamine for 12 min were washed (see MATERIALS AND METHODS) and subsequently assayed for [³H]dihydroergocryptine binding activity, the specific binding was restored to the level of the control (untreated) membranes (Fig. 1B). By contrast, when membranes exposed to 1 μM phenoxybenzamine for 12 min were washed under the same conditions as the

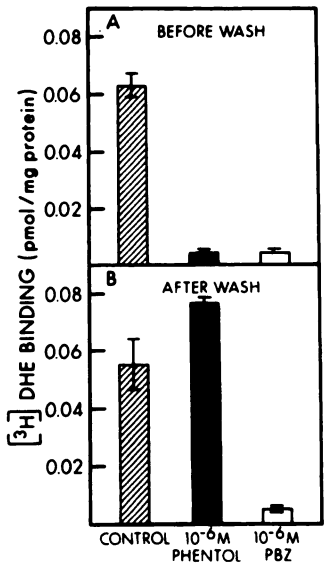

FIG. 1. *Effects of reversible and irreversible alpha adrenergic antagonists on specific [³H]dihydroergocryptine binding*

Rabbit uterine membranes (4 mg of protein per milliliter) were incubated with 1 μM phentolamine (phentol), 1 μM phenoxybenzamine (PBZ), or no added antagonist (control) as indicated, for 12 min at 25°. A. Specific [³H]dihydroergocryptine ([³H]-DHE) binding was assayed on the previously incubated membranes as described in MATERIALS AND METHODS. B. The incubated membranes were washed three times in buffer containing 50 mM Tris (pH 7.5) and 10 mM $MgCl_2$ by centrifugation and resuspension. The washed membranes were then assayed for [³H]dihydroergocryptine binding activity as in panel A. Each value is the mean ± standard error of triplicate determinations for two separate experiments.

phentolamine-treated membranes, the binding was not restored, but was apparently irreversibly blocked (Fig. 1B). Hence the blockade of binding sites by a 12-min incubation with phentolamine is easily reversible by washing, whereas the blockade of binding sites induced by a 12-min exposure to phenoxybenzamine is not reversible under these conditions.

Protection of alpha adrenergic receptor binding sites against phenoxybenzamine-induced irreversible blockade. It has been reported (2, 11) that the phenoxybenzamine-induced irreversible blockade of the contractile response to catecholamines can be prevented by the simultaneous presence of a competitive antagonist during the initial exposure of the muscle to phenoxybenzamine. Since this protection has been postulated to occur at the level of the *alpha* adrenergic receptor, studies were designed to test the ability of the competitive antagonist phentolamine to protect [^{3}H]dihydroergocryptine binding sites from irreversible inactivation by phenoxybenzamine. For these studies a partially blocking concentration of phenoxybenzamine (0.1 μM) was used to inactivate receptors, and a higher concentration (1 μM) of phentolamine was chosen as a protecting concentration, since 1 μM phentolamine reversibly occupies more than 90% of the receptor sites (Fig. 1). Membranes exposed to 0.1 μM phenoxybenzamine alone (12 min), followed by washing, possessed only 57% as many binding sites as control membranes (Fig. 2). By contrast, when 1 μM phentolamine was present during the preliminary incubation with 0.1 μM phenoxybenzamine, [^{3}H]dihydroergocryptine binding activity assayed following the membrane wash was equivalent to the control level of binding (Fig. 2). Hence the presence of the competitive antagonist phentolamine during the exposure to phenoxybenzamine completely protected the *alpha* adrenergic binding sites against irreversible inactivation by phenoxybenzamine.

Time course of irreversible receptor inactivation by phenoxybenzamine. The effects of phentolamine addition after various periods of phenoxybenzamine exposure were tested (Fig. 3). Membranes were exposed to 0.1 μM phenoxybenzamine for 12 min. At the indicated intervals after initiation of the incubation, a protecting concentration (1 μM) of phentolamine was added, and incubation was allowed to continue until a total of 12 min of exposure to phenoxybenzamine was completed. The membranes were then washed and [^{3}H]-dihydroergocryptine binding was assayed (Fig. 3). When phentolamine was added at the beginning of the incubation, the irreversible blockade induced by phenoxybenzamine was prevented. However, after 12 min of phenoxybenzamine exposure, the addition of phentolamine produced no effect on the irreversible inactivation of binding sites by phenoxybenzamine. When phentolamine was added at intermediate times between 0 and 12 min, partial protection against phenoxybenzamine inactivation was achieved. Thus the plot in Fig. 3 provides an estimate of the rate of development of irreversible noncompetitive inactivation of *alpha* receptors by 0.1 μM phenoxybenzamine in these membrane preparations. The half-time for

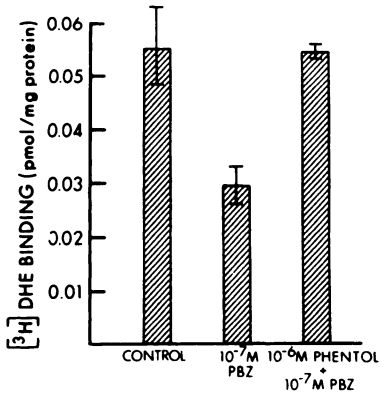

FIG. 2. *Protection of alpha adrenergic receptor binding sites against phenoxybenzamine-induced irreversible blockade*

Rabbit uterine membranes were incubated with no additions (control), 0.1 μM phenoxybenzamine (PBZ), or 0.1 μM phenoxybenzamine in the presence of 1 μM phentolamine (phentol) for 12 min at 25°. The membranes were then washed, and [^{3}H]dihydroergocryptine ([^{3}H]DHE) binding was assayed as described in Fig. 1B. Each value is the mean ± standard error of triplicate determination from two separate experiments.

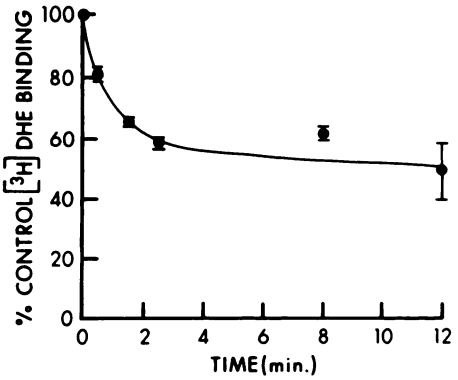

FIG. 3. *Time course of irreversible receptor inactivation by phenoxybenzamine*

Rabbit uterine membranes were incubated with 0.1 μM phenoxybenzamine for 12 min at 25°. At the indicated intervals after the beginning of the incubation, 1 μM phentolamine was added. After the completion of the 12-min incubation, the membranes were washed three times (see legend to Fig. 1B) and [^{3}H]dihydroergocryptine ([^{3}H]DHE) binding was assayed. Control binding refers to specific binding in the sample to which phentolamine had been added to the membranes just prior to the incubation with phenoxybenzamine. The abscissa represents the elapsed time of exposure to phenoxybenzamine before phentolamine was added.

this reaction is approximately 1 min. The maximal receptor-inactivating effect of 0.1 μM phenoxybenzamine was reached by 2.5 min of incubation.

Determination of number of receptor sites and apparent receptor affinity in the presence of beta adrenergic ligands. Membranes exposed to a reversible antagonist (0.1 μM phentolamine) or an irreversible antagonist (0.1 μM phenoxybenzamine) for 5 min at 25° were assayed for binding activity over a range of [^{3}H]dihydroergocryptine concentrations (Fig. 4). The binding in antagonist-treated and control membranes was compared by Scatchard analysis (Fig. 4). The negative reciprocal of the slope of each line is a measure of the apparent dissociation constant of the membrane receptors for [^{3}H]dihydroergocryptine, and the x intercept provides an estimate of the number of available [^{3}H]-dihydroergocryptine binding sites in the preparations. The results (Table 1) indicate that exposure to the irreversible *alpha* antagonist, phenoxybenzamine, leads to a significant reduction in the number of receptor binding sites, while the affinity of the unreacted sites for [^{3}H]dihydroergocryptine remains unchanged. By contrast, exposure of membranes to the reversible antagonist, phentolamine, results in a reduction in apparent affinity for [^{3}H]-dihydroergocryptine (higher dissociation constant for [^{3}H]dihydroergocryptine) without a significant reduction in number of binding sites. These results demonstrate the nonequilibrium nature of the inactivation of the binding sites by phenoxybenzamine, in contrast with the reversible competitive nature of the blockade produced by phentolamine. Ergotamine, an *alpha* adrenergic agonist with some antagonist activity for smooth muscle contraction, inhibited binding in a competitive manner (Fig. 5). The presence of 33 nM ergotamine resulted in a reduction in apparent affinity of the binding sites for [^{3}H]dihydroergocryptine. Thus the apparent K_D of [^{3}H]dihydroergocryptine binding was 4.4 times higher in the presence of ergotamine than in its absence. No change in receptor number (160 fmoles/mg of protein) was detectable in the presence of 33 nM ergotamine. Thus ergotamine competes for the binding sites in a manner similar to the competition for binding by the competitive antagonist phentolamine.

Data from Table 1 can be used to calculate the equilibrium dissociation constant of phentolamine for the binding sites, using the equation (12)

$$K_D = \frac{[\text{antagonist}]}{CR - 1}$$

where [antagonist] refers to the concentration of phentolamine (0.1 μM) and CR refers to the ratio of concentations of [^{3}H]dihydroergocryptine required to occupy the receptor sites half-maximally in the presence and absence of phentolamine (ratio of apparent K_D values for [^{3}H]dihydroergocryptine). The value obtained by this method (14 nM) is in good agreement with the value (15 nM) determined by directly measuring the ability of phentolamine to inhibit [^{3}H]dihydroergocryptine binding half-maximally (8).

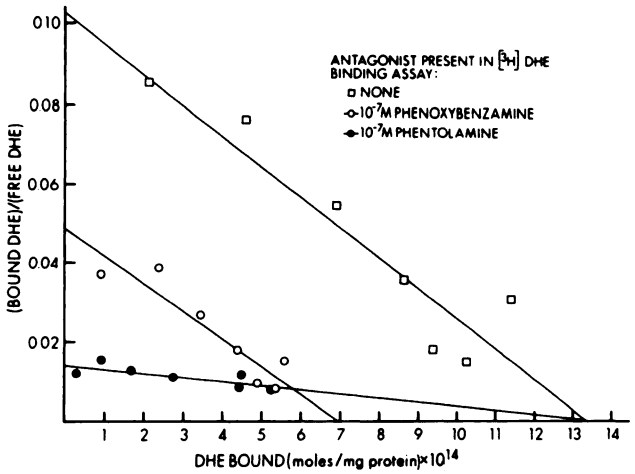

FIG. 4. *Scatchard analysis of [³H]dihydroergocryptine (DHE) binding in the presence and absence of alpha adrenergic antagonists*

Rabbit uterine membranes were incubated for 5 min at 25° in buffer containing 50 mM Tris (pH 7.5) and 10 mM $MgCl_2$ in the absence of antagonist (□), in the presence of 0.1 μM phenoxybenzamine (○), and in the presence of 0.1 μM phentolamine (●). Membranes were then used directly (without washing) in [³H]dihydroergocryptine binding assays designed for Scatchard analysis. Each point represents the mean of duplicate determinations. Lines were drawn by linear regression analysis. The experiment shown is representative of four such experiments (see Table 1).

Effects of sulfhydryl-modifying reagents on [³H]dihydroergocryptine binding. *Alpha* adrenergic responses can be inhibited by PHMB (5), an agent which reacts specifically with sulfhydryl groups. Accordingly we tested the effects of sulfhydryl-reactive agents on [³H]dihydroergocryptine binding sites. Uterine membranes were first incubated with PHMB (Table 2), and [³H]dihydroergocryptine binding was then assayed. Exposure of the membranes to 0.5 mM PHMB resulted in inhibition of 98% of the binding (Table 2). Inhibition of binding by PHMB was largely prevented by the simultaneous presence of 2 mM DTT, a sulfhydryl-containing compound. Moreover, when 2 mM DTT was added to the membranes subsequent to PHMB inactivation, the [³H]dihydroergocryptine binding activity was restored (Table 2). Hence DTT both blocks and reverses the inactivation of *alpha* adrenergic binding sites produced by PHMB. 2-Mercaptoethanol had little effect on specific [³H]dihydroergocryptine binding.

Effects of addition and removal of divalent cations on [³H]dihydroergocryptine binding. Since the presence of calcium is required for *alpha* adrenergic responses, the effects of divalent cations on [³H]dihydroergocryptine were tested (Table 3). The addition or removal of Ca^{2+} or Mg^{2+} does not affect the specific binding of [³H]dihydroergocryptine to rabbit uterine membranes. In addition, the presence of divalent cations does not alter the affinity of epinephrine for the *alpha* adrenergic receptor as assessed by the EC_{50} of epinephrine as an inhibitor of [³H]dihydroergocryptine binding (Table 3).

DISCUSSION

The radioligand binding techniques used in these investigations permit the direct study of the interaction of a variety of reagents with *alpha* adrenergic receptors. These studies demonstrate that the *alpha* antagonist phentolamine occupies *alpha* adrenergic receptor binding sites in a reversible competitive manner. By contrast, the haloalkylamine *alpha* adrenergic antagonist phenoxybenzamine irreversibly inactivates the *alpha* adrenergic

receptor binding sites. With phenoxybenzamine present at the low concentration of 0.1 μM, receptor site inactivation occurs at a rapid rate. The presence of a specific competitive *alpha* adrenergic antagonist (phentolamine) during phenoxybenzamine exposure of the membranes prevented irreversible receptor inactivation. This strongly suggests that phenoxybenzamine owes its biological irreversible blocking activity to its effect on the same specific *alpha* adrenergic receptor binding sites with which phentolamine acts. The mechanism of *alpha* receptor

TABLE 1

Effects of alpha adrenergic antagonists on number and apparent affinity of [³H]dihydroergocryptine binding sites

Experiments were performed as described for Fig. 4, and the data were derived from Scatchard plots. Lines were constructed by linear regression analysis of means of duplicate determinations. Values shown below are the means and standard errors of four experiments in which the density of binding sites (x intercept) and the apparent K_D of [³H]dihydroergocryptine for each binding site (negative reciprocal of the slope) were determined.

Antagonist	Density of [³H]dihydroergocryptine binding sites	Apparent K_D of [³H]dihydroergocryptine
	fmoles/mg protein	*nM*
None	130 ± 10	10.4 ± 1.3
Phentolamine, 0.1 μM	160 ± 34	73.0 ± 23[a]
Phenoxybenzamine, 0.1 μM	76 ± 10[b]	16.5 ± 3.9

[a] $p < 0.05$ compared with control.
[b] $p < 0.02$ compared with control.

TABLE 2

Effects of sulfhydryl-reactive reagents on specific [³H]dihydroergocryptine binding

Rabbit uterine membranes were incubated for 10 min at 25° in buffer (50 mM Tris, pH 7.5, and 10 mM $MgCl_2$) containing the reagents indicated. Specific [³H]dihydroergocryptine binding was then assayed as described under MATERIALS AND METHODS. Control binding (50–60 fmoles/mg of protein) refers to binding to a membrane preparation that had not been incubated with a group-specific reagent. Each value is the mean ± standard error of triplicate determinations in two experiments; where no standard error is shown, the variation from the mean in a single experiment was less than 10%.

Additon during preliminary incubation	Specific [³H]dihydroergocryptine binding
	% control
PHMB, 0.12 mM	95 ± 0
PHMB, 0.25 mM	61 ± 14
PHMB, 0.5 mM	2 ± 1
PHMB, 1.0 mM	0 ± 0
PHMB, 2.0 mM	0 ± 0
PHMB, 2.0 mM and DTT, 2.0 mM	86 ± 3
DTT, 2.0 mM	88 ± 1
PHMB, 2.0 mM, then DTT, 2.0 mM[a]	105
2-Mercaptoethanol, 2.0 mM	92

[a] Two 10-min preliminary incubations were done, the first with PHMB and the second with DTT.

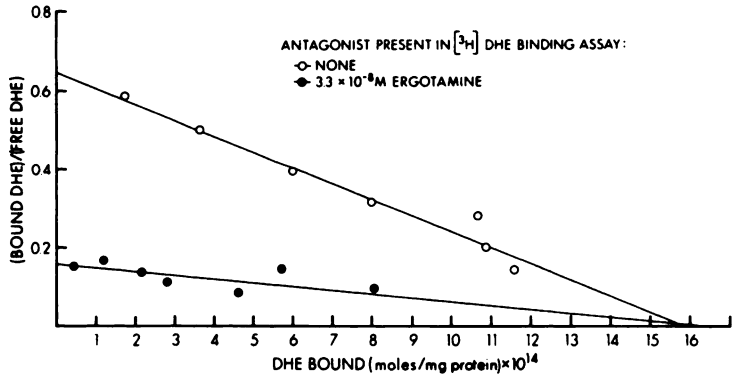

FIG. 5. *Scatchard analysis of [³H]dihydroergocryptine (DHE) binding in the presence (●) and absence (○) of ergotamine*

The Experimental design was the same as in the legend to Fig. 4.

TABLE 3

[^{3}H]dihydroergocryptine binding in the presence and absence of divalent cations

Rabbit uterine membranes were prepared as described under MATERIALS AND METHODS, except that they were washed twice in 50 mM Tris buffer, pH 7.5, containing no magnesium. After suspension in the buffers indicated below, all at pH 7.5, the membranes were incubated with 8 nM [^{3}H]dihydroergocryptine, and specific binding was determined as described under MATERIALS AND METHODS. The negative logarithms of the concentrations of epinephrine causing half-maximal inhibition (pD_2) were also determined as a measure of the affinity of epinephrine for the binding sites. Values are the means of triplicate determinations, which varied less than 10% from the means.

Buffer	Specific [^{3}H]dihydroergocryptine binding	pD_2 for inhibition of [^{3}H]dihydroergocryptine binding by epinephrine
	fmoles/mg protein	
50 mM Tris-HCl	67	6.77
50 mM Tris-HCl + 2 mM EGTA[a]	70	6.77
50 mM Tris-HCl + 3.3 mM Ca^{2+}	69	6.64
50 mM Tris-HCl + 10 mM Mg^{2+}	77	6.46

[a] EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

binding site inactivation by phenoxybenzamine probably involves covalent bond formation with an essential group at the active site of the *alpha* receptor (13). The chemical nature of the covalent bond formed between binding site and phenoxybenzamine is not known at this time. It has been proposed that phenoxybenzamine can alkylate sulfhydryl groups (5, 13). Data presented here (Table 2) suggest that the *alpha* adrenergic binding site does possess an essential sulfhydryl group. Thus phenoxybenzamine may well inactivate the *alpha* adrenergic receptor by alkylating this essential sulfhydryl group.

The inactivation of *alpha* adrenergic receptors by 0.1 μM phenoxybenzamine was very rapid ($t_{1/2}$ ~1 min) and was incomplete (Fig. 3). The lack of complete receptor inactivation by 0.1 μM phenoxybenzamine may possibly be due to a rapid loss of free phenoxybenzamine in the incubation solution. If there were no loss of free phenoxybenzamine, 0.1 μM phenoxybenzamine should be a concentration sufficient to inactivate totally the relatively low concentation (0.7–1.0 nM) of *alpha* adrenergic receptors present. The loss of free phenoxybenzamine might be due either to reaction with other sites or to hydrolysis in the solution. Higher concentrations (1 μM) of phenoxybenzamine were sufficient to inactivate virtually all of the *alpha* adrenergic receptors (Fig. 1).

The results of these direct binding studies (Figs. 3 and 4 and Table 1) provide a more accurate and quantitative assessment of the action of phenoxybenzamine at the *alpha* adrenergic receptor sites than has been provided by the results of previous pharmacological experiments. Although pharmacological experiments have been used for estimating the fraction of *alpha* adrenergic receptors inactivated in intact tissues on exposure to β-haloalkylamines (4), the procedure employed is based on analysis of dose-response data with an equation derived from a model. The model allows for the possibility of spare receptors, but since it contains a number of inherent theoretical assumptions, some of which cannot be checked experimentally, the estimates of fractional inactivation of receptors obtained with the pharmacological procedure must be accepted with some caution. In contrast to the indirect pharmacological procedure, the radioligand binding studies reported here give a direct measure of the reduction in number of *alpha* adrenergic receptors produced by exposure to low concentrations of phenoxybenzamine and show that the reduction in number of receptors occurs without a significant change in apparent receptor affinity. In these direct binding studies, the existence of potential spare receptors does not complicate the analysis of nonequilibrium blockade of binding sites.

Neither the addition nor the removal of calcium affects the number of active *al-*

pha adrenergic binding sites or their affinity for adrenergic agonists (Table 3). Hence the reported calcium dependence of *alpha* adrenergic responses (6) is clearly not due to a calcium requirement for the binding of agonist to receptor.

These studies demonstrate the utility of direct [³H]dihydroergocryptine binding studies in elucidating the mechanisms by which pharmacological agents alter *alpha* adrenergic responses. Phentolamine is clearly a competitive *alpha* adrenergic antagonist which interacts directly and reversibly with the receptor. Phenoxybenzamine directly inactivates receptor binding in an irreversible, noncompetitive manner. Similarly, the sulfhydryl reagent PHMB acts directly on the receptor. Divalent cations, however, act at steps in the *alpha* adrenergic response other than the receptor.

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