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"Spare" *Alpha*$_1$-Adrenergic Receptors and the Potency of Agonists in Rat Vas Deferens

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SUMMARY

The existence of "spare" *alpha*$_1$-adrenergic receptors in rat vas deferens was examined directly using radioligand binding assays and contractility measurements. *Alpha*$_1$-adrenergic receptors in homogenates of rat vas deferens were labeled with [^{125}I]BE 2254 (^{125}IBE). Norepinephrine and other full *alpha*$_1$-adrenergic receptor agonists were much less potent in inhibiting ^{125}IBE binding than in contracting the vas deferens *in vitro*. Treatment with 300 nM phenoxybenzamine for 10 min to irreversibly inactivate *alpha*$_1$-adrenergic receptors caused a large decrease in the potency of full agonists in causing contraction of this tissue and a 23–48% decrease in the maximal contraction observed. Using those data, equilibrium constants for activation (K_{act} values) of the receptors by agonists were calculated. These K_{act} values agreed well with the equilibrium binding constants (K_D values) determined from displacement of ^{125}IBE binding. The reduction in *alpha*$_1$-adrenergic receptor density following phenoxybenzamine treatment was determined by Scatchard analysis of specific ^{125}IBE binding sites and compared with the expected reduction (q values) calculated from the agonist dose-response curves before and after phenoxybenzamine treatment. Exposure to 300 nM phenoxybenzamine for 10 min resulted in a 39% decrease in specific ^{125}IBE binding sites, which did not agree with the 93% decrease expected from the calculated q values. Treatment of vas deferens with a dose of phenoxybenzamine (10 μM for 15 min) that completely abolished the contractile response to *alpha*$_1$-adrenergic agonists caused an 82% decrease in the density of ^{125}IBE binding sites. Tissues exposed to 300 nM phenoxybenzamine in the presence of 100 μM phentolamine or 3 μM prazosin showed no change in the dose-response curves for agonist-induced contraction or in the density of ^{125}IBE binding sites when compared with controls. This suggests that phenoxybenzamine functionally inactivates *alpha*$_1$-adrenergic receptors at or near the receptor binding site. These experiments suggest that the potencies of agonists in activating *alpha*$_1$-adrenergic receptors in rat vas deferens agree well with their potencies in binding to the receptors. The greater potency of agonists in causing contraction may be due to spare receptors in this tissue. The data also demonstrate that phenoxybenzamine irreversibly inactivates *alpha*$_1$-adrenergic receptors in rat vas deferens, but that the decrease in receptor density is much smaller than that predicted from receptor theory.

INTRODUCTION

A basic assumption of receptor theory is that occupation of a receptor by an agonist may not be linearly related to the response of the tissue (1–4). In many tissues it has been proposed that there are more receptors than are necessary to provoke a maximal functional response, and that a maximal response may be obtained with only a small fraction of the receptors occupied. The remaining receptors are "spare" in that they do not contribute to the maximal response of the tissue, although these "spare" receptors will increase the apparent potency of agonists in causing a tissue response. If spare receptors are present, by the law of mass action, a smaller proportion of receptors would need to be occupied to produce a response. Therefore, lower concentrations of agonist would be needed to occupy the receptors, and a response would occur at a lower concentration of drug. This would be manifested by a progressive shift to the left of the agonist dose-response curve in the presence of increasing percentages of spare receptors (1–4).

Although the concept of spare receptors is fundamental to an understanding of drug action, the experimental evidence supporting it is limited. The theory was introduced in 1956 by Stephenson (1) based on unexpected variations in the potencies of a series of muscarinic

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receptor agonists of different hydrocarbon chain lengths on contractions of the guinea pig ileum. In the same year Nickerson (2) provided more direct evidence for the spare receptor theory by using the nonequilibrium antagonist dibenamine to irreversibly block histamine receptors in the guinea pig ileum. He found that dibenamine caused a large shift to the right in the dose-response curve for histamine before any significant decrease in the maximal contraction was observed (2). Other investigators subsequently used dibenamine and related compounds, such as phenoxybenzamine to provide similar evidence for spare muscarinic, histaminergic, and *alpha*$_1$-adrenergic receptors in a variety of tissues (3–5). However, since it has only recently become possible to measure receptor density using radioligand binding assays, these investigators were unable to prove that the non-equilibrium antagonists they used actually inactivated the receptors. Although several recent studies have used radioligand binding to examine directly spare muscarinic and *beta*-adrenergic receptors in heart and smooth muscle (6–8), the results have not been conclusive. Some of the evidence has supported the existence of spare receptors in these tissues: however, a substantial variation is often noticed when the observed data are compared with data predicted from receptor theory (6, 7). Thus the experimental evidence supporting the spare receptor theory is still equivocal.

We have recently used ^{125}IBE[1] (9, 10) to label *alpha*$_1$-adrenergic receptors in homogenates of rat vas deferens, and compared the binding properties of these receptors with the pharmacological properties of the *alpha*$_1$-adrenergic receptor-mediated contraction *in vitro* (11). An excellent correlation was observed between the potency of antagonists in inhibiting ^{125}IBE binding and their pA$_2$ values for competitively inhibiting the contractile response to agonists. However, full agonists were much more potent in initiating a contractile response than in competing for the ^{125}IBE binding site, possibly because of the existence of spare receptors in this tissue (11). In this study we use phenoxybenzamine to irreversibly inactivate *alpha*$_1$-adrenergic receptors and to determine whether spare *alpha*$_1$-adrenergic receptors are present in rat vas deferens.

METHODS

Materials The drugs used were obtained from the following sources: norepinephrine bitartrate, epinephrine bitartrate, and phenylephrine HCl, Sigma Chemical Company (St. Louis, Mo.); *d,l*-α-methylnorepinephrine, (+)-norepinephrine, and (+)-epinephrine, Sterling-Winthrop (New York, N. Y.); methoxamine, Burroughs Wellcome (Research Triangle Park, N. C.); and phenoxybenzamine HCl, Smith Kline & French Laboratories (Philadelphia, Pa).

Measurement of contractile response. Male Sprague-Dawley rats (175–300 g), pretreated with reserpine (3 mg/kg i.p.) were killed, and the entire vas deferens from each rat was removed and placed in Krebs-Ringer bicarbonate buffer (KRB buffer) containing 120 mM NaCl, 5.5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 20 mM $NaHCO_3$, 11 mM glucose, and 0.029 mM $CaNa_2$ EDTA, gassed with 95% O_2/5% CO_2. Desmethylimipramine (0.1 μM), 1 μM *d,l*-propranolol, and 1 μM normetanephrine were added as described previously (11). After removing the adherent sheath, each vas deferens was suspended in a 10-ml organ bath maintained at 37° and attached to a Grass FT.03 force displacement transducer. After a 1-hr equilibrium period at a resting tension of 500 mg, the tissues were exposed twice to a maximal concentration of norepinephrine and thoroughly washed.

Agonist-induced increases in isometric tension were determined using a noncumulative dosage schedule with 6-min intervals between doses. Responses were measured as the peak tension developed while the preparation was exposed to the agonist and are expressed as a percentage of the maximal contraction induced by norepinephrine or phenylephrine.

Exposure to phenoxybenzamine. After performing the initial dose-response curves for agonist-induced contractions, preparations were washed three times. Five minutes later, phenoxybenzamine was added, and at the end of the exposure period the tissues were washed three times successively and then six times over the next 30 min. Ten minutes later, agonist dose-response curves were repeated. For determination of the density of ^{125}IBE binding sites, preparations were prepared as usual for measurement of contraction, and the above procedure was followed exactly, except that the agonist dose-response curves both before and after phenoxybenzamine treatment were omitted. Control tissues were treated in the same manner but were not exposed to phenoxybenzamine.

Measurement of ^{125}IBE 2254 binding. A single vas deferens was homogenized in 20 ml of 20 mM $NaPO_4$ buffer (pH 7.6) containing 154 mM NaCl (PO_4-salt buffer) with a Polytron at speed 7 for 15 sec and centrifuged at 20,000 × *g* for 10 min; the pellet was resuspended in 3.5 ml of PO_4-salt buffer and filtered through a double layer of surgical gauze to remove connective tissue fragments (11). BE 2254 was radioiodinated as described by Engel and Hoyer (9). Scatchard analysis of specific ^{125}IBE binding was performed by incubating 100 μl of tissue homogenate (20–40 μg of protein) with six concentrations of ^{125}IBE (15,000–500,000 cpm; 25–800 pM)/20 mM $NaPO_4$ (pH 7.6)/154 mM NaCl in the presence or absence of 10 μM phentolamine in a final volume of 250 μl for 20 min at 37°. At the end of the incubation period, each tube was diluted with 10 ml of 10 mM Tris-Cl (pH 7.4) at 20° and immediately poured over a glass-fiber filter (Schleicher and Schuell No. 30) under reduced pressure. Each filter was washed with 10 ml of 10 mM Tris-HCl (pH 7.4) and counted in a *gamma* counter. Nonreceptor binding was determined to be binding in the presence of 10 μM phentolamine, and was usually about 20–30% of total binding (11).

Data analysis. All data analysis was performed on an Apple II Plus computer. EC_{50} values of dose-response curves for increases in isometric tension were determined by linear regression of all points between 20% and 80% of the maximal response to that agonist. Scatchard plots were analyzed by nonweighted linear regression.

Equilibrium activation constants for agonists (K_{act} values) were determined from the dose-response curves before and after fractional receptor inactivation with phenoxybenzamine by the method of Furchgott (4), using the equation

$$\frac{1}{[A]} = \frac{1}{q} \cdot \frac{1}{[A']} + \frac{1-q}{q \cdot K_{act}} \qquad (1)$$

where $[A]$ is the concentration of agonist necessary to give a fixed percentage of the maximal response before phenoxybenzamine treatment, $[A']$ is the concentration of agonist necessary to give the same percentage of the maximal response after phenoxybenzamine treatment, K_{act} is the equilibrium activation constant for the agonist, and q is the fraction of the initial receptor concentration remaining after phenoxybenzamine treatment. A plot of $1/[A]$ against $1/[A']$ should give a straight line with a slope of $1/q$ and an intercept of $(1-q)/(q \cdot K_{act})$ (4). For each experiment, the agonist dose-response curves before and after phenoxybenzamine treatment were analyzed by linear regression of all points between 20% and 80% of the maximal response obtained in each case. Using the linear functions obtained in this manner, the dose of agonist necessary to give 30, 40, 50, 60, and 70% of the maximal response before and after phenoxybenzamine treatment were calculated. Linear regression of the reciprocals of these calculated values resulted in K_{act} and q values as described above.

[1] The abbreviation used is: ^{125}IBE, ^{125}I-labeled BE 2254.

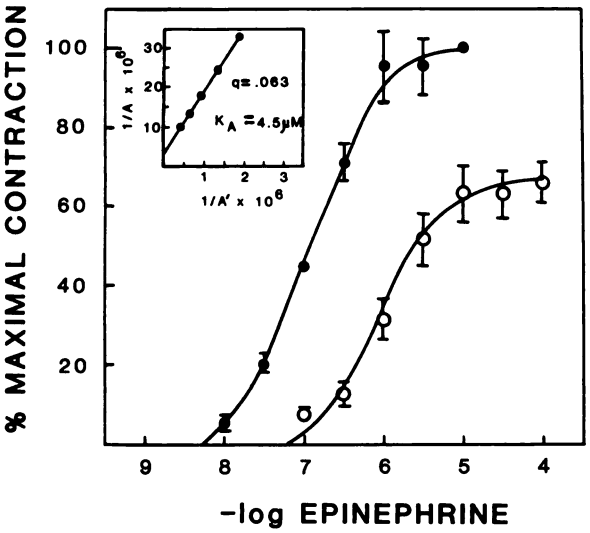


FIG. 1. *Effect of phenoxybenzamine treatment on epinephrine-induced contractions of rat vas deferens*

Tissues were mounted in organ baths and equilibrated as described in the text. Epinephrine was added in a noncumulative fashion at the indicated doses (●) and maximal contractile tension was recorded. Tissues were then exposed to 300 nM phenoxybenzamine for 10 min and, after washout, the dose-response curve to epinephrine was repeated (○). Each point represents the mean ± standard error of the mean from four different preparations. Drug concentrations are molar. *Inset*, Plot of reciprocals of equieffective drug concentrations before ($1/A$) and after ($1/A'$) phenoxybenzamine treatment. K_A and q values were calculated as described in the text.

RESULTS

Effect of phenoxybenzamine treatment on agonist-induced contractions of rat vas deferens. Dose-response curves for agonist-induced increases in isometric tension in rat vas deferens were determined before and after exposure to 300 nM phenoxybenzamine for 10 min. Phenoxybenzamine treatment caused a 10-fold shift to the right in the dose-response curve for (−)-epinephrine-induced contraction of rat vas deferens and a 35% decrease in the maximal contraction obtained (Fig. 1). Similar results were obtained with (−)-norepinephrine, phenylephrine, methoxamine, (±)-α-methylnorepinephrine, (+)-epinephrine, and (+)-norepinephrine (Table 1; Fig. 2). In each case, treatment with 300 nM phenoxybenzamine for 10 min caused a 10- to 30-fold decrease in the potency of the agonist in inducing a contractile response, and a 23%–48% decrease in the maximal contractile response.

Determination of K_{act} values for agonists. Equilibrium activation constants (K_{act} values) were calculated for each agonist using the dose-response curves obtained before and after phenoxybenzamine treatment as described by Furchgott (4). Plotting the reciprocals of equieffective agonist concentrations before and after phenoxybenzamine treatment gave a straight line (*inset* to Fig. 1) from which a K_{act} value could be calculated as (slope -1)/y intercept. K_{act} values obtained in this manner for each of the seven full agonists are shown in Table 2. There was an excellent correlation between these K_{act} values and the binding constants (K_D values) for agonists at *alpha*$_1$-adrenergic receptors in vas deferens (obtained from the competitive inhibition of ^{125}IBE binding to particulate preparations of vas deferens) (Table 2). As shown previously (11), without any receptor inactivation agonists are much more potent in causing contraction of the vas deferens than they are in occupying the receptor (Table 1). However, when spare receptors are presumably inactivated by phenoxybenzamine treatment, the calculated K_{act} values are in good agreement with the binding constants for agonists.

Calculation of the theoretical percentage of functional receptors remaining after phenoxybenzamine treatment (q values). The theoretical percentage of functional receptors remaining after phenoxybenzamine treatment (q values) can also be calculated as 1/slope of the plot of reciprocals of equieffective agonist concentrations before and after phenoxybenzamine treatment (4). The calculated theoretical percentage of receptors remaining after exposure to 300 nM phenoxybenzamine for 10 min was similar for each agonist and ranged from 3% to 12% remaining (Table 3).

Direct measurement of receptor density after phenoxybenzamine treatment. In order to examine directly the effects of phenoxybenzamine treatment on *alpha*$_1$-adrenergic receptor density in rat vas deferens, Scatchard analysis of ^{125}IBE binding was performed on control vas deferens and vas deferens treated for 10 min with 300 nM phenoxybenzamine (Table 4; Fig. 3). This concentration of phenoxybenzamine caused a 39% ± 6% decrease (n = 8) in the B_{max} for ^{125}IBE in vas deferens membranes with no change the K_D for ^{125}IBE. Thus,

TABLE 1

Effect of phenoxybenzamine on EC_{50} values and maximal contraction induced by agonists in rat vas deferens

Dose-response curves for agonists were performed before and after exposure to 300 nM phenoxybenzamine for 10 min. EC_{50} values were calculated by linear regression of all points between 20% and 80% of the maximal response. Each value is the mean ± standard error of the mean of the indicated number of experiments.

Agonist	EC_{50} before phenoxybenzamine	EC_{50} after phenoxybenzamine	Maximal response after phenoxybenzamine	N
	μM	*μM*	*% control*	
(−)-Epinephrine	0.12 ± 0.003	1.2 ± 0.20	65 ± 4.8	4
(−)-Norepinephrine	0.35 ± 0.067	10.2 ± 3.93	52 ± 5.5	9
Phenylephrine	0.86 ± 0.17	26.4 ± 11.5	62 ± 0.8	4
(±)-α-Methylnorepinephrine	1.5 ± 0.17	16.2 ± 3.1	77 ± 4.2	4
Methoxamine	11.6 ± 3.95	166 ± 58.8	56 ± 0.9	4
(+)-Epinephrine	11.7 ± 3.5	239 ± 60.0	55 ± 1.0	4
(+)-Norepinephrine	10.9 ± 0.7	227 ± 48.7	58 ± 0.6	4

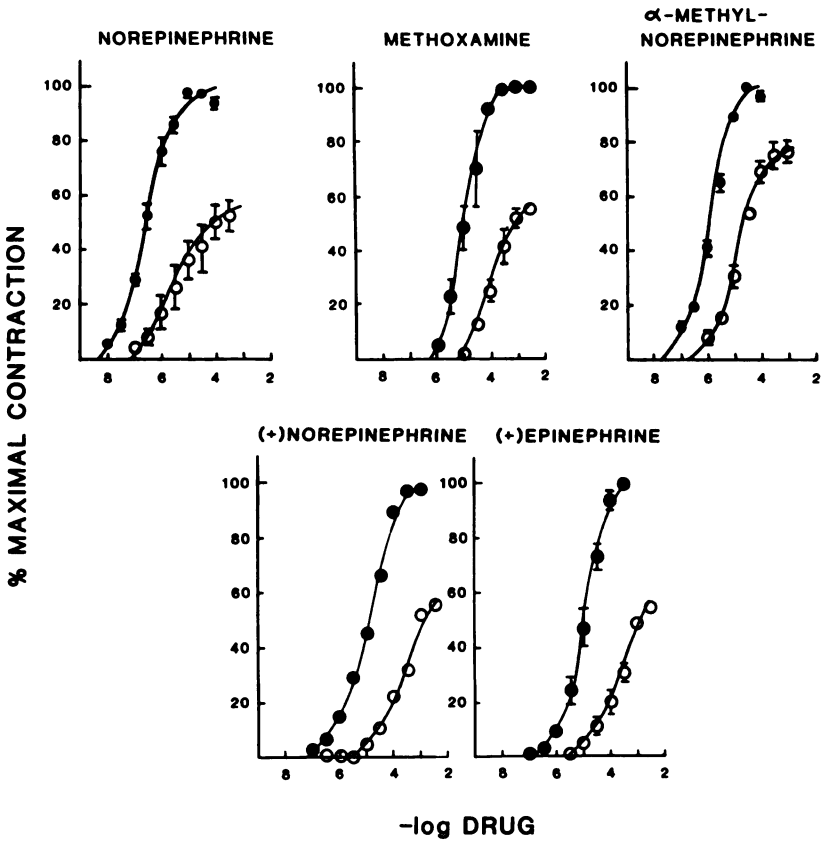


FIG. 2. *Effect of phenoxybenzamine treatment on contractions of rat vas deferens induced by (−)-norepinephrine, methoxamine, (±)-α-methylnorepinephrine, (+)-norepinephrine, and (+)-epinephrine*

Experimental details as described in Fig. 1. Each point represents the mean ± standard error of the mean of data from four to nine preparations. Drug concentrations are molar.

although phenoxybenzamine did inactivate *alpha*$_1$-adrenergic receptor binding sites in rat vas deferens, this inactivation (39%) was much smaller than that predicted from theory (93%). To determine whether there was a population of ^{125}IBE binding sites in rat vas deferens that could not be inactivated by phenoxybenzamine, other vasa deferentia were exposed to 10 μM phenoxybenzamine for 15 min. This concentration completely abolished the contractile response to phenylephrine (Fig. 3) and other *alpha*$_1$-adrenergic receptor agonists (data not shown) and caused an 82% loss in the density of ^{125}IBE binding sites with no change in the K_D value (Table 4).

TABLE 2

Comparison of K_D values for binding and calculated K_{act} values for alpha$_1$-adrenergic receptor agonists in rat vas deferens

K_D values for the inhibition of ^{125}IBE binding to membranes from rat vas deferens were determined as previously described (11). K_{act} values were determined by measuring dose-response curves for agonist-induced contraction before and after 300 nM phenoxybenzamine treatment for 10 min. K_{act} values were calculated as (slope−1)/y intercept from a plot of reciprocals of equieffective drug concentrations before and after phenoxybenzamine treatment. Each value is the mean ± standard error of the mean of three to nine independent determinations.

Drug	K_D	K_{act}	Ratio (K_D/K_{act})
	μM	μM	
(−)-Epinephrine	7.4 ± 0.5	2.9 ± 1.4	2.55
(−)-Norepinephrine	11.2 ± 0.8	6.3 ± 2.2	1.78
Phenylephrine	13.1 ± 1.1	38.0 ± 14.2	0.35
(±)-α-Methylnorepinephrine	44.5 ± 17.1	45.7 ± 16.3	0.97
Methoxamine	72.9 ± 6.7	83.9 ± 8.8	0.87
(+)-Epinephrine	65.1 ± 11.5	133 ± 19.4	0.49
(+)-Norepinephrine	218 ± 37.1	216 ± 51.8	1.01

Possible contribution of alpha$_2$-adrenergic receptors to contractile response following phenoxybenzamine treatment. It was possible that a previously undetected population of postsynaptic *alpha*$_2$-adrenergic receptors contributed to the contractile-response of rat vas deferens following inactivation of the *alpha*$_1$-receptors with phenoxybenzamine. To test this possibility, vasa deferentia were treated with 300 nM phenoxybenzamine for 10 min and washed, and a methoxamine dose-response curve was determined in the absence or presence of the *alpha*$_2$-selective blocker yohimbine at a concentration of 1 μM (Fig. 4). Yohimbine had no effect on the methoxamine-induced contractile response (Fig. 4), suggesting that *alpha*$_2$-adrenergic receptors do not contribute to this response.

Protection against phenoxybenzamine by phentolamine and prazosin. It was possible that phenoxybenzamine inactivates *alpha*$_1$-adrenergic receptors at a site other than, or in addition to, the drug binding site. Experiments were performed to determine whether occupation

TABLE 3

Percentage of alpha$_1$-adrenergic receptors remaining in rat vas deferens following exposure to 300 nM phenoxybenzamine for 10 min, as measured by different methods

Dose-response curves for agonist-induced contraction were determined before and after exposure to 300 nM phenoxybenzamine for 10 min. q Values were calculated as 1/slope of a plot of reciprocals of equieffective drug concentrations before and after phenoxybenzamine treatment. % Receptors remaining was calculated as (100 · q). Direct measurement of ^{125}IBE binding sites was done by Scatchard analysis as described in the legend to Table 4. Each value is the mean ± standard error of the mean of the indicated number of observations.

Method	% Receptor remaining	N
Indirect		
q Values calculated from dose-effect curves before and after phenoxybenzamine using		
(−)-Norepinephrine	11.6 ± 4.3	9
(−)-Epinephrine	8.4 ± 2.9	4
Phenylephrine	2.9 ± 0.7	4
Methoxamine	8.6 ± 1.6	4
(±)-α-Methylnorepinephrine	7.5 ± 0.7	4
(+)-Epinephrine	5.6 ± 1.5	4
(+)-Norepinephrine	3.3 ± 0.7	4
Direct		
Scatchard analysis of ^{125}IBE binding sites	61.4 ± 6.0	8

of the receptor binding site with the *alpha*-adrenergic antagonist phentolamine, or the *alpha*$_1$-adrenergic selective antagonist prazosin, would protect the receptors against inactivation by phenoxybenzamine. Tissues were exposed to 300 nM phenoxybenzamine for 10 min in the presence of either 100 μM phentolamine or 3 μM prazosin, and dose-response curves for contraction by phenylephrine were measured. Phentolamine or prazosin was also added to control tissues to control for incomplete washout of drug. The potency of phenylephrine was the same in tissues exposed to phenoxybenzamine in the presence of either 100 μM phentolamine (data not shown) or 3 μM prazosin (Fig. 5) as it was in control tissues which had been incubated with phentolamine or prazosin alone. Exposure of control tissues to either phentolamine or prazosin alone caused a large shift to the right in the dose-response curve to phenylephrine, probably due to incomplete washout of the antagonist. The presence of 3 μM prazosin during phenoxybenzamine exposure also completely blocked the effect of phenoxybenzamine on ^{125}IBE binding sites (Fig. 5). Thus occupation of the receptors by prazosin will protect against both functional inactivation of the receptors by phenoxybenzamine and inactivation of the receptor binding sites by phenoxybenzamine.

TABLE 4

Effect of phenoxybenzamine treatment on the density of ^{125}IBE binding sites in rat vas deferens

Vasa deferentia from reserpinized rats were desheathed and equilibrated for 1 hr at 37° at 500 mg of resting tension in Krebs-Ringer bicarbonate buffer. One vas from each animal was treated with phenoxybenzamine as indicated; the other served as an untreated control. After washout of the phenoxybenzamine, tissues were removed from the organ bath and homogenized, and Scatchard analysis of ^{125}IBE binding was performed as described in the text. Each value is the mean ± standard error of the mean of eight tissues from two different experiments.

	B_{max}	K_D
	fmoles/mg protein	*pM*
Control	189 ± 9.8	215 ± 17.1
Phenoxybenzamine (300 nM) for 10 min	116 ± 11.4 (61%)	215 ± 27.6
Control	229 ± 23.6	182 ± 26.4
Phenoxybenzamine (10 μM) for 15 min	41 ± 8.9 (18%)	234 ± 45.3

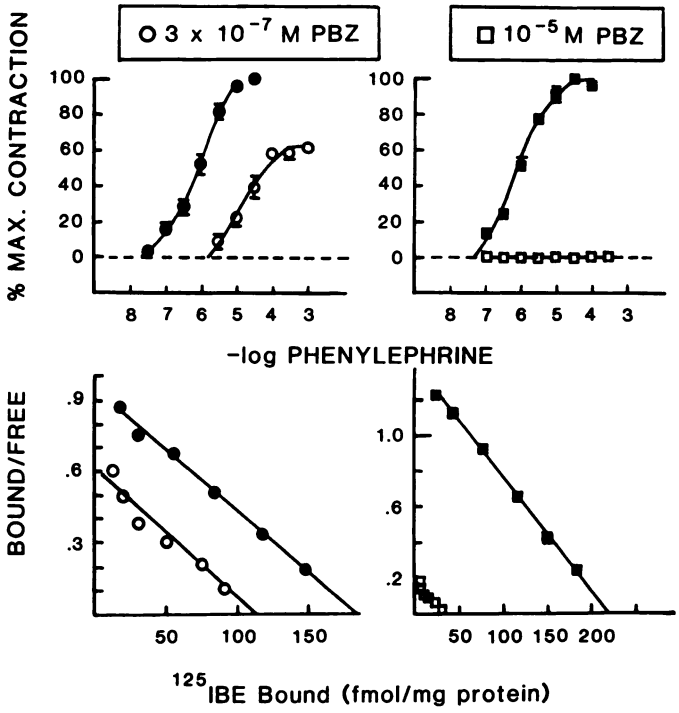


FIG. 3. *Effect of phenoxybenzamine (PBZ) on phenylephrine-induced contractions and specific ^{125}IBE binding sites in rat vas deferens*

Top. Vasa deferentia were mounted in organ baths and equilibrated, and noncumulative dose-response curves for increases in isometric tension by phenylephrine (●, ■) were determined. Tissues were then exposed to 300 nM phenoxybenzamine for 10 min (○) or 10 μM phenoxybenzamine for 15 min (□) and dose-response curves to phenylephrine were repeated. Each point represents the mean ± standard error of the mean of eight determinations from two separate experiments. Drug concentrations are molar.

Bottom. Vasa deferentia were mounted in organ baths as for contractile measurements above, and exposed to either no phenoxybenzamine (●, ■), 300 nM phenoxybenzamine for 10 min (○) or 10 μM phenoxybenzamine for 15 min (□). After washout, tissues were removed from the organ bath and homogenized, and Scatchard analysis of specific ^{125}IBE binding was determined as described in the text. Each point represents the mean of eight determinations from two separate experiments.

DISCUSSION

^{125}IBE labels a homogeneous population of binding sites in homogenates of rat vas deferens, and we have shown that antagonists and partial agonists compete for these sites with potencies identical with their potencies in inhibiting or activating the *alpha*$_1$-adrenergic receptor-

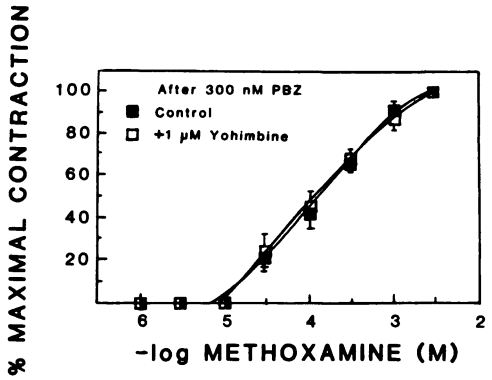


FIG. 4. *Effect of yohimbine on alpha-adrenergic receptor-mediated contractions of rat vas deferens following phenoxybenzamine (PBZ) treatment*

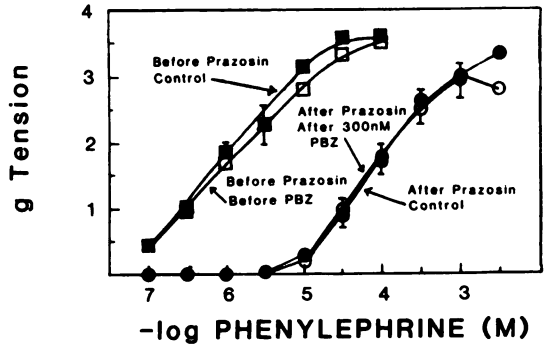
Tissues were mounted in organ baths, equilibrated, and exposed to 300 nM phenoxybenzamine for 10 min as described in the text. Following washout of the phenoxybenzamine, a dose-response curve to methoxamine was determined (■). The tissue was then equilibrated with 1 μM yohimbine for 45 min, and another dose-response curve to methoxamine was determined (□). Each point represents the mean ± standard error of the mean of four determinations.

mediated contractile response in intact vas deferens (11). In contrast, full agonists are 10- to 100-fold more potent in activating contraction of rat vas deferens than in competing for the ^{125}IBE binding sites (11). We here present evidence that this discrepancy between the potency of agonists in binding and causing contraction is probably due to the existence of a pool of spare *alpha*$_1$-adrenergic receptors in this tissue.

In the presence of spare receptors, receptor theory predicts that full agonists will be more potent in activating a half-maximal tissue response than in occupying half of the total receptor pool, since only a small proportion of the receptors needs to be occupied to obtain a maximal tissue response (1–3). Treatment with an irreversible antagonist to inactivate the spare receptor pool should then decrease the potency of the agonist in activating a response (2). Eventually, the limiting case is reached whereby the dose-response curve for activating the tissue response is superimposable on the dose-response curve for binding to the receptor (4). Using this experimental manipulation, one can then calculate equilibrium activation constants (K_{act} values) for agonists, which should be identical with the K_D values of the agonists in binding to the receptors (4). We have shown here that treatment of intact rat vas deferens with phenoxybenzamine caused a 10- to 30-fold decrease in the potency of all full agonists in activating a contractile response in this tissue. K_{act} values calculated from these experiments agreed well with the K_D values of agonists in binding to the receptors. This evidence supports the hypothesis that the greater potency of *alpha*$_1$-adrenergic receptor agonists in activating contraction of rat vas deferens than in binding to the *alpha*$_1$-adrenergic receptors is due to the presence of spare receptors in this tissue.

The agreement between the calculated K_{act} values and the K_D values for the binding of agonists to *alpha*$_1$-adrenergic receptors in rat vas deferens provides direct experimental evidence in support of the methods for determination of K_{act} values. Although these methods are an extrapolation of receptor theory (4), they have not previously been subjected to experimental confirmation. The results presented here suggest that these calculated K_{act} values are true measures of the binding constants for agonists.

Examination of the K_{act} values calculated for various agonists at postjunctional, contractile *alpha*-adrenergic receptors in various tissues reveals a surprisingly wide range of values. K_{act} values reported for norepinephrine ranged from 0.13 μM in rabbit aorta (12) to 6.3 μM in rat vas deferens (present study). Similar variations have



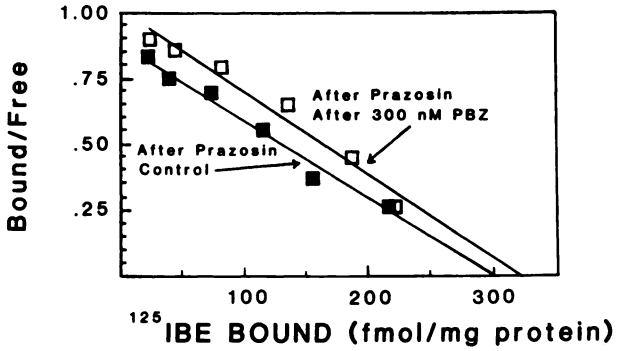


FIG. 5. *Protection against the effects of phenoxybenzamine (PBZ) by pretreatment with prazosin*

Vas deferens were mounted in organ baths and equilibrated, and dose-response curves to phenylephrine were determined as described in the text. Prazosin was then added to all baths at a final concentration of 3 μM for 30 min. Phenoxybenzamine (300 nM) was then added to some baths for 10 min, and all baths were washed three times within 5 min. Prazosin was then washed out of all baths (six washes over 30 min), and a dose-response curve to phenylephrine was again determined. At the end of the dose-response curve, each vas deferens was homogenized in 10 ml of PO_4-salt buffer, centrifuged, and resuspended in 3.5 ml of PO_4-salt buffer. Scatchard analysis of specific ^{125}IBE binding was determined as described in the text.

Top. Dose-response curves for contraction of intact vas deferens by phenylephrine before (■, □) and after (●, ○) treatment with 3 μM prazosin. Each point represents the mean ± standard error of the mean of three determinations. Bottom. Scatchard analysis of specific ^{125}IBE binding on a particulate fraction of rat vas deferens after prazosin treatment alone (■) or after treatment with 300 nM phenoxybenzamine in the presence of 3 μM prazosin (□). Each point represents the mean of the three determinations.

been observed for other agonists and have been suggested to represent a heterogeneity of the *alpha*-adrenergic receptors in various tissues (12–14). Most of these receptors appear to be mainly of the $alpha_1$ subtype, and it is thought that the variations in agonist K_{act} value may represent subtypes of $alpha_1$-adrenergic receptors, although it is possible that there may be some $alpha_2$-adrenergic receptors contributing to some of these responses (16).

It is interesting, however, that not all of the predictions of receptor theory were observed to be fulfilled in these studies. Direct measurement of receptor density by Scatchard analysis of ^{125}IBE binding showed that the density of $alpha_1$-adrenergic receptors in rat vas deferens was reduced following phenoxybenzamine treatment. However, the observed reduction in receptor binding sites (39%) was much less than that predicted from the theoretical q values (93%). The large majority of studies supporting the concept of spare receptors have been carried out with phenoxybenzamine or related drugs such as dibenamine (2–6). These drugs inactivate several types of receptors, including histaminergic (2) and muscarinic cholinergic (5, 6) as well as *alpha*-adrenergic receptors. However, the great potency and rate of receptor inactivation observed with these compounds, as well as the ability to protect selectively against receptor inactivation by occupation of the binding site with either agonists or antagonists (2–5), have suggested that these agents act by directly alkylating the receptor binding site. It has been assumed, although not previously directly tested, that the effects of phenoxybenzamine and dibenamine on agonist dose-response curves in isolated tissues are proportional to the site-directed direct alkylation of these receptors.

We found that treatment of intact rat vas deferens with phenoxybenzamine caused a decrease in the density of $alpha_1$-adrenergic receptor binding sites similar to that observed previously in homogenized tissues (17, 18). This supports the hypothesis that phenoxybenzamine inactivates the receptor in a manner such that drugs no longer bind to it with high affinity. However, the percentage decrease in the density of ^{125}IBE binding sites was much smaller than the percentage decrease in the functional receptors predicted from the calculated q values. There are two major possible explanations for this discrepancy: (a) phenoxybenzamine is inactivating the receptors (or signal transduction mechanism) at a site which does not affect drug binding to the receptor, or (b) the $alpha_1$-adrenergic receptors mediating the contractile response represent a small percentage of the total receptor pool, and are inactivated at a faster rate than the remaining receptors.

Since occupation of the $alpha_1$-adrenergic receptors in rat vas deferens by phentolamine or prazosin completely blocked the effects of phenoxybenzamine on phenylephrine-induced contractions and ^{125}IBE binding sites, it seems unlikely that phenoxybenzamine is inactivating the receptor at a site other than the binding site. Occupation of the receptor by phentolamine or prazosin might trigger a conformational change in the receptor molecule such that phenoxybenzamine can no longer inactivate it; however, since these drugs are antagonists and do not trigger receptor-mediated responses, there is no evidence that such a conformational change occurs. In addition, since phenoxybenzamine clearly does inactivate the receptor binding sites, if there were also inactivation at other sites it would have to occur at a faster rate than inactivation of the binding site, which would be unusual for a site-directed agent.

It is possible that not all of the specific binding sites labeled by ^{125}IBE are functional $alpha_1$-adrenergic receptors in rat vas deferens. We have shown, however, that the potencies of antagonists, partial agonists, and full agonists in competing for ^{125}IBE binding sites are indistinguishable from their potencies at the $alpha_1$-adrenergic receptors mediating contraction in this tissue (ref. 11 and present study). The Hill coefficients of all of these compounds, except for 3-imidazoline compounds, in competing for the binding sites are close to 1.0, suggesting that the population of binding sites labeled by ^{125}IBE in rat vas deferens is virtually homogeneous with regard to its binding properties for drugs (11). Since a high dose of phenoxybenzamine (10^{-5} M for 15 min) inactivates more than 80% of the specific ^{125}IBE binding sites, it is unlikely that there is a population of ^{125}IBE binding sites insensitive to phenoxybenzamine inactivation. Therefore, if binding sites exist which are not functional receptors, they cannot yet be distinguished on the basis of their binding properties.

The discrepancy between the predicted loss of functional receptors caused by phenoxybenzamine treatment and the observed loss of ^{125}IBE binding sites could be due to access barriers in the intact muscle (19). If the $alpha_1$-adrenergic receptors mediating the contractile response were localized on the surface of the outer longitudinal muscle, then it would be expected that these receptors would be inactivated preferentially. The remaining receptors might be on the inner circular muscle protected from inactivation by the outer longitudinal muscle layer. Receptors on the circular muscle would probably not contribute significantly to longitudinal contractions. Alternatively, it is possible that there are "silent" $alpha_1$-adrenergic receptors not linked to a signal transduction mechanism in rat vas deferens, and that their localization (i.e., intracellular, intramembrane) makes them resistant to inactivation by phenoxybenzamine. These possibilities are currently being explored.

It is interesting to note that previous studies which have examined the concept of spare receptors directly using radioligand binding assays have also obtained results which deviate significantly from those predicted by theoretical considerations. Venter (7), using a covalent antagonist to inactivate *beta*-adrenergic receptors in cat heart ventricle, found a 7-fold shift to the right in the inotropic dose-response curve to isoproterenol associated with only a 43% decrease in *beta*-adrenergic receptor density, whereas theoretical considerations would predict a greater than 85% loss in receptor density. Similarly, Takeyasu *et al.* (6) used dibenamine to inactivate muscarinic receptors in guinea pig ileum and found that direct measurements of muscarinic receptor density with radioligand binding assays deviated significantly from the predicted q values (65% inactivation of binding sites and 92% inactivation of functional receptors). Thus,

direct experimental examination of the phenomenon of spare receptors has not yielded completely convincing results. As Waud (20) pointed out, although the spare receptor concept seems likely *a priori*, direct experimental proof is still needed.

In summary, we have presented evidence that exposure of rat vas deferens to phenoxybenzamine decreased the potency of agonists in causing contraction of this tissue. Calculation of agonist equilibrium activation constants (K_{act} values) from these data show good agreement with the binding constants (K_D values) for these drugs obtained with competitive radioligand binding techniques, supporting the existence of a pool of spare *alpha*-adrenergic receptors in this tissue. We have shown that phenoxybenzamine treatment irreversibly inactivates *alpha*$_1$-adrenergic receptors in rat vas deferens; however, the observed decrease in receptor density is much smaller than that predicted from theory. The reason for this discrepancy is not yet known.

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