MOLECULAR PHARMACOLOGY, 27:517–524

Nonlinear Relationship between α_1-Adrenergic Receptor Occupancy and Norepinephrine-Stimulated Calcium Flux in Cultured Vascular Smooth Muscle Cells

WILSON S. COLUCCI, TOMMY A. BROCK, MICHAEL A. GIMBRONE, JR., AND R. WAYNE ALEXANDER

The Cardiovascular Division and the Vascular Pathophysiology Laboratory, Departments of Medicine and Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Received September 20, 1984; Accepted February 14, 1985

SUMMARY

To determine the relationship between vascular α_1-adrenergic receptor occupancy and receptor-coupled calcium flux, we have studied [^{3}H]prazosin binding and *l*-norepinephrine-induced ^{45}Ca efflux in cultured vascular smooth muscle cells isolated from the rabbit aorta. In a crude cellular homogenate, [^{3}H]prazosin bound to a single high affinity site (K_d = 0.096 nM; B_{max} = 105 ± 15 fmol/mg of protein), whereas *l*-norepinephrine (NE) binding was best described by a two-site model with 43 ± 8% of sites of high affinity (K_H = 92 ± 3 nM) and 57 ± 7% of sites of low affinity (K_L = 7460 ± 4330 nM). NE-stimulated ^{45}Ca efflux was concentration-dependent (EC_{50} = 108 nM) and potently inhibited by prazosin (IC_{50} = 0.15 nM), but not yohimbine (no inhibition at 10 μM). For the total receptor pool identified by [^{3}H]prazosin binding, the relationship between receptor occupancy by NE and NE-stimulated ^{45}Ca efflux was markedly nonlinear, such that 50% of maximum NE-stimulated efflux occurred with occupancy of only approximately 7% of receptors. Likewise, following irreversible inactivation of 69 ± 5% of receptors by phenoxybenzamine, maximal NE-stimulated ^{45}Ca efflux was decreased by only 8 ± 2%. These two experimental approaches provide direct evidence for the presence in cultured rabbit aortic smooth muscle cells of a sizable pool of α_1-adrenergic receptors in excess of those needed for maximum NE-stimulated ^{45}Ca efflux. This evidence of "spare" receptors, together with the finding of two affinity states of agonist binding, raises the possibility of functional heterogeneity of α_1-adrenergic receptors in this system.

INTRODUCTION

The modulation of vascular α-adrenergic responsiveness can occur at one or more loci, including the receptor itself, its coupling to intermediate biochemical events involved in calcium mobilization, or more distal steps associated with contractile protein function. Through the use of radioligand-binding assays which allow direct characterization and quantification of adrenergic receptors, it has been demonstrated that both the number and affinity of vascular α_1-adrenergic receptors can be modulated by a variety of factors including agonist exposure, catecholamine depletion, heterotropic hormones, and guanine nucleotides (1–4).

However, since little is known about the relationship between vascular α-adrenergic receptor occupancy and physiologic responses, it has not been possible to assess the importance of such changes in α-adrenergic receptor number and affinity in determining vascular responsiveness to α-adrenergic stimulation. For instance, if the relationship between receptor occupancy and contractile response were linear, an alteration in receptor number would result in a change in the maximum amplitude of response, whereas a change in receptor affinity would result in a shift in sensitivity (5). Alternately, if "spare" receptors were present in excess of the number necessary for a maximal response, a change in receptor number could result in either a change in maximum amplitude or sensitivity, depending on the magnitude of the alteration in receptor number and the number of spare receptors (5). Furthermore, it is possible that alterations in physiologic responsiveness may be mediated by postreceptor mechanisms, and therefore may occur in the absence of any change in receptor number or affinity (6, 7).

Previous studies of the α-adrenergic receptor occupancy-response relationship in rabbit aorta, performed by comparing α-adrenergic-induced contraction in the presence and absence of irreversible receptor blockade with phenoxybenzamine, have provided inferential evidence for the presence of spare receptors in excess of the

This work was supported in part by National Institutes of Health Grants HL-29763 and HL-22602. W. S. C. is a Clinician-Scientist of the American Heart Association.

0026-895X/85/050517-08$02.00/0

number required for a maximal contractile response (8, 9). However, more recent evaluations of the spare receptor hypothesis by use of radioligand methods to directly determine receptor occupancy in rat vas deferens (10, 11) and canine aorta (12) have not been entirely consistent with this hypothesis.

The current study was designed to delineate the relationship between α-adrenergic receptor occupancy and a closely coupled physiologic response, NE[1]-stimulated calcium efflux. For this purpose, we have utilized cultured rabbit aortic smooth muscle cells which retain α_1-adrenergic receptors (3) and receptor-coupled ^{45}Ca efflux (4). The occupancy-response relationship was evaluated by two methods. First, the magnitude of maximal NE-stimulated ^{45}Ca efflux was compared to the fractional occupancy of α-adrenergic receptors by NE, as determined by analysis of NE competition for the [^{3}H]prazosin-binding site. Second, maximal NE-stimulated ^{45}Ca efflux was determined in cells pretreated with phenoxybenzamine to irreversibly inactivate (8, 11, 13) approximately 70% of the α-adrenergic receptors detected by [^{3}H]prazosin binding.

MATERIALS AND METHODS

Cell culture. Rabbit aortic vascular smooth muscle cells were cultured by a modification of an enzymatic dissociation method previously reported by our laboratory (14). Briefly, male New Zealand White rabbits weighing 2 to 3 kg were sacrificed by cervical dislocation, and the thoracic aortas were excised under sterile conditions. The adventitia, intima, and outer third of the medial layer were dissected away and discarded. The remaining portion of the medial layer was finely minced and incubated for 90 min at 37° with gentle shaking in an enzyme digestion mixture consisting of medium 199 (M. A. Bioproducts, Walkersville, MD), elastase, (0.25 mg/ml; pancreatopeptidase, EC 3.4.4.7; type III; 90–100 units/mg, Sigma Chemical Co.), soybean trypsin inhibitor (0.75 mg/ml; type I-S, Worthington Biochemical Corp.), collagenase (2.0 mg/ml; *Clostridium histolyticum*, C1S type I, Worthington Biochemical Corp.), and crystallized bovine serum albumin (1.0 mg/ml; Miles Laboratories). The resulting digest was sieved through a 100-μm stainless steel mesh (Bellco Glass Corp., Vineland, NJ). The fragments retained by the sieve were triturated 10 times through a 14-gauge stainless steel cannula (Bard Parker) and resieved to separate the remaining fragments from dispersed cells. These isolated cells were pelleted by centrifugation (200 × *g*, 5 min) in a siliconized conical glass tube and plated in one or two 75-cm^2 culture flasks (Falcon) in medium 199 supplemented with L-glutamine, antibiotics, and 20% heat-inactivated fetal calf serum (M. A. Bioproducts). The cultures were washed at 24 hr to remove debris, and refed at 1- to 3-day intervals. Primary cultures were grown to confluence (approximately 7 to 10 days) and then passaged after brief exposure to trypsin-EDTA. All experiments presented here utilized cells from several strains between passage levels 3 and 30 which typically had grown for 7 to 14 days after plating at 1×10^4 cells/cm^2.

[^{3}H]Prazosin binding. Cells for [^{3}H]prazosin binding were seeded and grown to confluence in 100-mm plastic culture dishes (Falcon). Each dish was washed three times with isotonic NaCl (4°), after which the cells were scrape-harvested with a rubber policeman, and pelleted by centrifugation (500 × *g*, 10 min, 4°). The pellet was resuspended in hypotonic buffer solution (Tris, 5 mM; MgCl, 1 mM; pH 7.5; 4°) for 10 min and processed in a Dounce homogenizer with 10 strokes of a Falcon tissue Dounce A-pestle. The crude homogenate was resuspended in assay buffer (Tris, 50 mM; $MgCl_2$, 10 mM; pH 7.5) to a final protein concentration of approximately 0.3 to 0.6 mg/ml.

The binding assay was performed in a total volume of 1.0 ml consisting of assay buffer (0.3 ml), [^{3}H]prazosin in assay buffer (0.1 ml), competing drug in assay buffer (0.1 ml), and crude tissue homogenate (0.5 ml). Agonist and antagonist competition binding experiments utilized a [^{3}H]prazosin concentration of approximately 1 nM. The assay mixture was incubated for 60 min at 30°, filtered through a Whatman GF/C glass fiber filter, and washed three times with room temperature assay buffer (4.5 ml/wash). The filters were dried and counted in liquid scintillation fluid. All data refer to specific binding, which was defined as the counts displaced by 100 nM prazosin.

^{45}Ca efflux. Cells for calcium efflux experiments were replicate plated in 35-mm plastic culture dishes. Prior to assay, confluent monolayers were equilibrated for 18 to 24 hr with 2 ml of fresh culture medium containing 4 μCi of ^{45}Ca (Amersham, Chicago) to ensure labeling of the cellular pools of exchangeable calcium. Cells were preincubated with *dl*-propranolol (2 μM; 15–30 min; 37°) just prior to the start of the assay in order to block the effects of norepinephrine on β-adrenergic receptors. ^{45}Ca efflux was initiated by washing the cultures three times with BSS containing (mM): NaCl, 130; KCl, 5; MgCl, 1.0; CaCl, 1.5; glucose, 10; and HEPES, 20 (buffered to pH 7.4 with Tris base) and adding 1 ml of BSS containing 1 mg/ml of bovine serum albumin with or without *l*-norepinephrine. At various time intervals, the reaction was terminated by washing the cultures four times with ice-cold, Ca^{2+}-free BSS containing 10 mM LaCl, followed by an additional 5-min incubation with 2 ml of this same solution. The combination of La^{3+} and cold temperature blocks Ca^{2+} influx, retards Ca^{2+} efflux, and displaces Ca^{2+} from external binding sites (15). Cell-associated ^{45}Ca was then determined by extracting the cells with 1 ml of 0.1 N HNO_3 (20 min, 4°) and counting the radioactivity. Nonspecific binding was determined under identical conditions using cell-free dishes. Cellular ^{45}Ca content was expressed as nanomoles/mg of protein and was calculated from the specific activity of ^{45}Ca in the culture medium. Initial cellular ^{45}Ca content averaged 5.20 ± 0.70 nmol/mg of protein, and the net decrease in ^{45}Ca content after 6-min exposure to 10 μM NE (0.67 ± 0.06 nmol/mg of protein) averaged 13.0 ± 1% of the initial isotopic content (n = 12). Experiments were performed in triplicate or quadruplicate, and replicates agreed within 5%.

Phenoxybenzamine treatment of cells. In order to irreversibly inactivate α-adrenergic receptors (8, 11, 13), phenoxybenzamine was added to the growth medium to achieve a final concentration of 1 nM and incubated for 90 min at 37°. Prior to harvesting for [^{3}H]prazosin binding or initiation of ^{45}Ca efflux, culture dishes were washed three times with serum-free medium 199 (37°) or BSS, respectively.

Analysis of data. Saturation binding curves for [^{3}H]prazosin and drug competition curves for the [^{3}H]prazosin-binding site were analyzed by computerized nonlinear curve fitting using the LIGAND program as described by Munson and Rodbard (16). The fractional α-adrenergic receptor occupancy by various concentrations of *l*-norepinephrine was computed by Eq. 1, based on the laws of mass action (17), in which B = concentration of bound NE, K_d = dissociation constant for NE determined by nonlinear curve fitting of NE competition for the [^{3}H]prazosin-binding site, R = total receptor concentration, and T = total concentration of NE:

$$B = \frac{(K_d + R + T) - [(K_d + R + T)^2 - 4RT]^{1/2}}{2} \quad (1)$$

All data are presented as the mean ± standard error, and comparisons of group means were made by nonpaired t test (two-tailed).

Chemicals. Phenoxybenzamine was a gift of Smith, Kline and French, Inc. All other chemicals were obtained from Sigma, or as previously described (1, 4).

RESULTS

Binding of [^{3}H]prazosin to rabbit aortic smooth muscle cell homogenate. The binding of [^{3}H]prazosin to crude

[1] The abbreviations used are: NE, norepinephrine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BBS, balanced salt solution; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

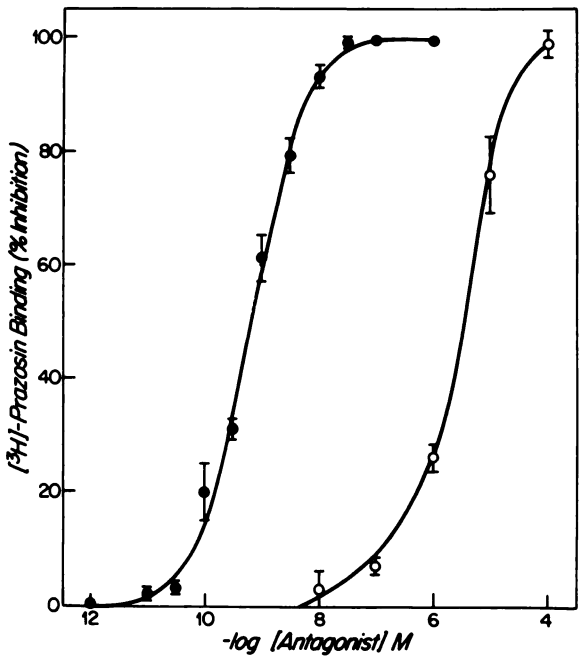

FIG. 1. *Competition of prazosin (●) and yohimbine (○) for the [^{3}H] prazosin-binding site in homogenized rabbit aortic smooth muscle cells*

The curves were steep with Hill coefficients of 1, and were best fit by a one-site binding model. Data depicted are the means ± standard error of five (prazosin) or three (yohimbine) experiments performed in duplicate.

homogenate from rabbit aortic smooth muscle cells was of high affinity and saturable over a [^{3}H]prazosin concentration range of 0.01 to 10 nM. Computerized nonlinear curve fitting (LIGAND) indicated that binding was best described by a one-site model ($p < 0.001$ versus a two-site model). In six experiments, the mean dissociation constant (K_d) was 0.096 ± 0.025 nM, and the mean density of binding sites (B_{max}) was 105 ± 15 fmol/mg of protein. Under the assay conditions utilized, the binding of [^{3}H]prazosin reached equilibrium by 30 min and was completely dissociated following exposure to an excess (100 nM) of unlabeled prazosin (data not shown).

Competition for the [^{3}H]prazosin-binding site by agonists and antagonists. As previously described, the potency order of agonists and antagonists in competing for the [^{3}H]prazosin-binding site was that of an α_1-adrenergic receptor (4). For agonists, the order of potency was *l*-epinephrine ≥ *l*-norepinephrine ≫ *l*-isoproterenol, and for antagonists the order was prazosin > phentolamine > yohimbine. The *levo*-isomer of epinephrine was 13-fold more potent than the *dextro*-isomer in competing for the [^{3}H]prazosin-binding site, thus indicating that the binding of [^{3}H]prazosin was appropriately stereospecific.

Antagonist competition curves were steep with slope factors of unity (Fig. 1), and computer analysis of binding by nonlinear curve fitting indicated that the competition curves for prazosin, phentolamine, and yohimbine were most consistent with a one-site model ($p < 0.001$ versus a two-site model). The K_d for unlabeled prazosin determined in this manner (0.055 nM; $n = 5$) was in good agreement with the K_d determined from saturation binding experiments. The K_d for yohimbine was 124 nM ($n = 3$).

In contrast to antagonist competition curves, agonist competition curves for the [^{3}H]prazosin-binding site were shallow with slope factors less than unity. The NE competition curve is delineated in detail in Fig. 2. The mean concentration of NE that inhibited binding by 50% was 9900 nM, and the mean K_d determined by nonlinear curve fitting to a one-site model was 963 nM. In seven experiments, the mean slope factor for NE was 0.61, and nonlinear curve fitting of the combined data was most consistent with a two-site model ($p < 0.001$ versus a one-site model) in which 41% of sites were of high affinity (K_d = 75 nM) and 59% were of a lower affinity (K_d = 2670 nM). When the binding curves were analyzed individually, a two-site model provided a significantly better fit ($p < 0.05$) than a one-site model in six of the seven experiments. Analyzed in this way (i.e., individually), the mean K_d for the high affinity site was 92 ± 3 nM (43 ± 8% of sites), and for the low affinity site was 7460 ± 4330 nM (57 ± 7% of sites). The results of NE competition curves performed at a lower [^{3}H]prazosin concentration of 0.23 nM were similar to those at a [^{3}H]prazosin concentration of 1 nM with a mean Hill coefficient of 0.57, and a mean K_d of 1.23 μM ($n = 3$). Addition of the nonhydrolyzable guanine nucleotide analog Gpp(NH)p (1 mM) to the assay buffer had no apparent effect on the NE competition curve ($n = 2$; data not shown).

NE-stimulated ^{45}Ca efflux. We previously demonstrated that NE stimulates a rapid increase in ^{45}Ca efflux (onset < 30 sec) from cultured rabbit aortic cells (4). Norepinephrine increased ^{45}Ca efflux in a concentration-

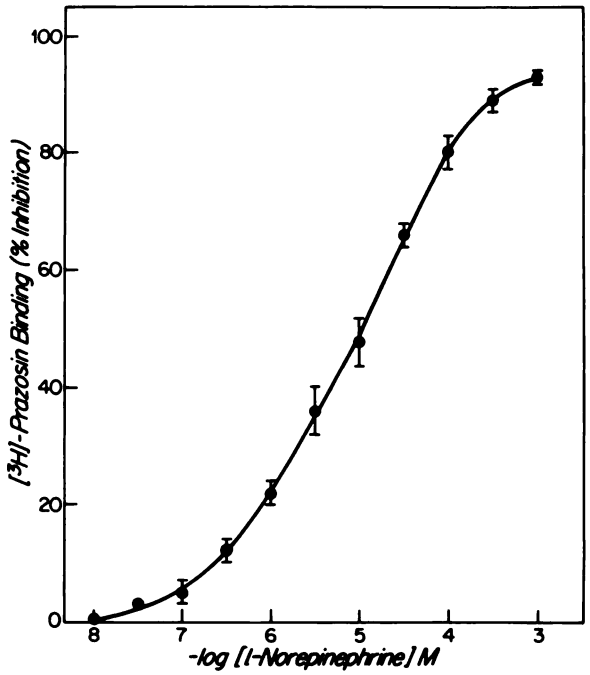

FIG. 2. *Competition of l-norepinephrine for the [^{3}H]prazosin-binding site*

Compared to antagonist competition curves, the *l*-norepinephrine curves were less steep, and nonlinear curve fitting was most consistent with a two-site binding model. The data depicted are the mean ± standard error for six experiments performed in duplicate.

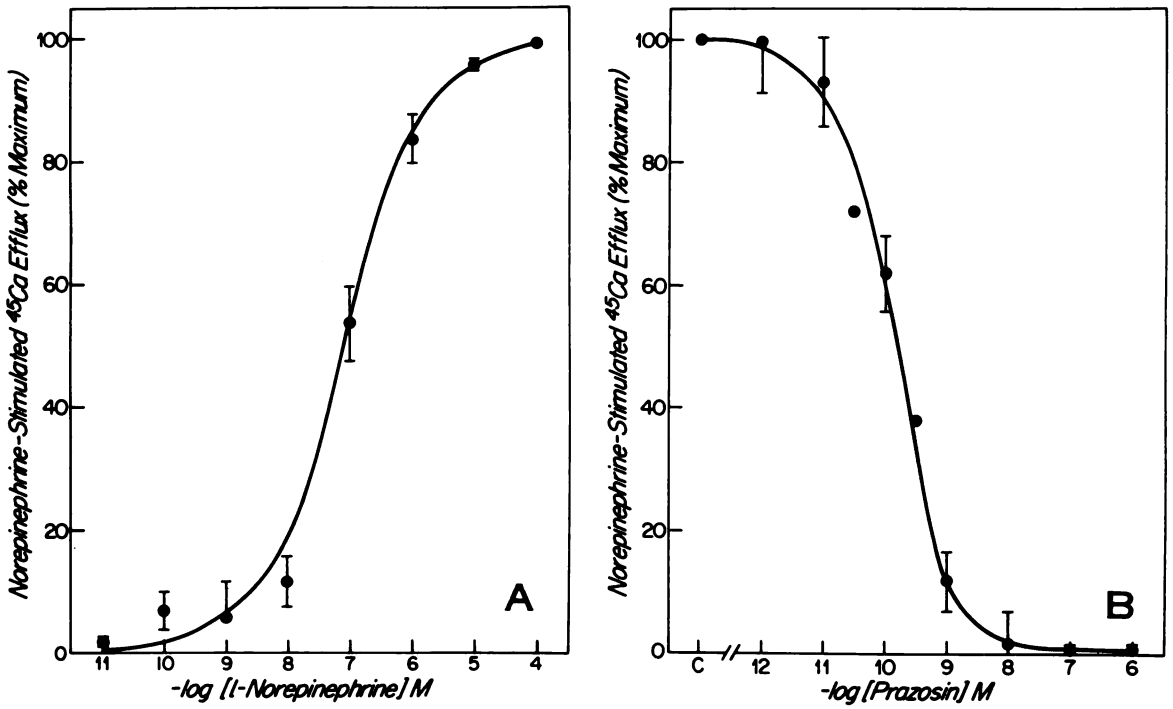

FIG. 3. *Stimulation and inhibition of ^{45}Ca efflux*

A, norepinephrine stimulation of unidirectional ^{45}Ca efflux from cultured rabbit aortic smooth muscle cells. Norepinephrine-stimulated ^{45}Ca efflux was calculated as the difference in ^{45}Ca content of preloaded cells in the presence or absence of the indicated concentrations of norepinephrine (see Materials and Methods). Initial cellular ^{45}Ca content averaged 5.20 ± 0.70 nmol/mg of protein, and the net decrease in ^{45}Ca content at t = 6 min in response to 10 μM NE (0.67 ± 0.06 nmol/mg of protein) averaged 13 ± 1% of the initial isotope content (n = 12). Data depicted are the mean ± standard error for six experiments performed in triplicate. B, prazosin inhibition of ^{45}Ca efflux stimulated by 10 μM norepinephrine. Cells were incubated with the indicated concentrations of prazosin for 30 min prior to initiation of ^{45}Ca efflux. Data indicate the mean ± standard error of three experiments performed in triplicate.

dependent manner (Fig. 3A). The mean effective concentration (EC_{50}) for NE was 108 nM (n = 7 experiments), and the concentrations required to elicit threshold and maximum responses were approximately 1 nM and 100 μM, respectively.

The increase in ^{45}Ca efflux by 10 μM NE was completely blocked by 100 nM prazosin (Fig. 3B). Prazosin inhibition of NE-stimulated efflux was concentration-dependent, with a threshold of approximately 0.01 nM and a mean inhibitory concentration (IC_{50}) of 0.15 nM (n = 4 experiments). The α_2-selective antagonist yohimbine had no effect on NE-stimulated ^{45}Ca efflux at concentrations up to 100 nM (data not shown).

Receptor occupancy versus NE-stimulated ^{45}Ca efflux. To delineate quantitatively the relationship between receptor occupancy by NE and NE-stimulated ^{45}Ca efflux, fractional receptor occupancy by NE was plotted against the percentage of maximum NE-stimulatable ^{45}Ca efflux over a NE concentration range of 0.01 nM to 0.1 mM (Fig. 4A). A hyperbolic function was described such that 50% of maximum ^{45}Ca efflux occurred when only approximately 7% of the total receptor pool was occupied by NE (Fig. 4B). In contrast, when ^{45}Ca efflux was plotted against the fractional occupancy of only high affinity NE-binding sites that account for about 43% of the total receptor pool (as determined by nonlinear curve fitting), the relationship was more nearly linear with a coefficient of linear regression of 0.99 (Fig. 4B).

Effect of phenoxybenzamine on [^{3}H]prazosin binding and maximal NE-stimulated ^{45}Ca efflux. Exposure of cells to phenoxybenzamine (1 nM, 90 min, 37°) resulted in a consistent 69 ± 5% (n = 6) reduction in [^{3}H]prazosin-binding sites (Fig. 5). As previously described (13), the decrease in binding sites was not accompanied by a change in [^{3}H]prazosin-binding affinity (K_d = 0.24 nM; n = 2). Likewise, NE competition curves following phenoxybenzamine treatment were shallow with a similar K_d to control experiments (Hill coefficient = 0.53; K_d = 1.16 μM; n = 2). In cells treated with phenoxybenzamine in this manner, maximal NE-stimulated ^{45}Ca efflux in response to 10 μM NE was reduced only slightly to 92 ± 2% of control (n = 6) (Fig. 5).

DISCUSSION

[^{3}H]Prazosin binding to α_1-adrenergic receptors. The binding site for [^{3}H]prazosin identified in the rabbit aortic smooth muscle cell exhibits the characteristics of an α_1-adrenergic receptor. Binding is of high affinity, saturable, reversible, and of the appropriate potency order in competition experiments with agonists and antagonists. The approximately 2000-fold higher potency of prazosin relative to the α_2-selective ligand yohimbine in competing for binding, along with the steep competition curves for both drugs, indicates that [^{3}H]prazosin is labeling only α_1 receptors. In addition, the lack of saturable binding by the α_2-selective radioligand [^{3}H]

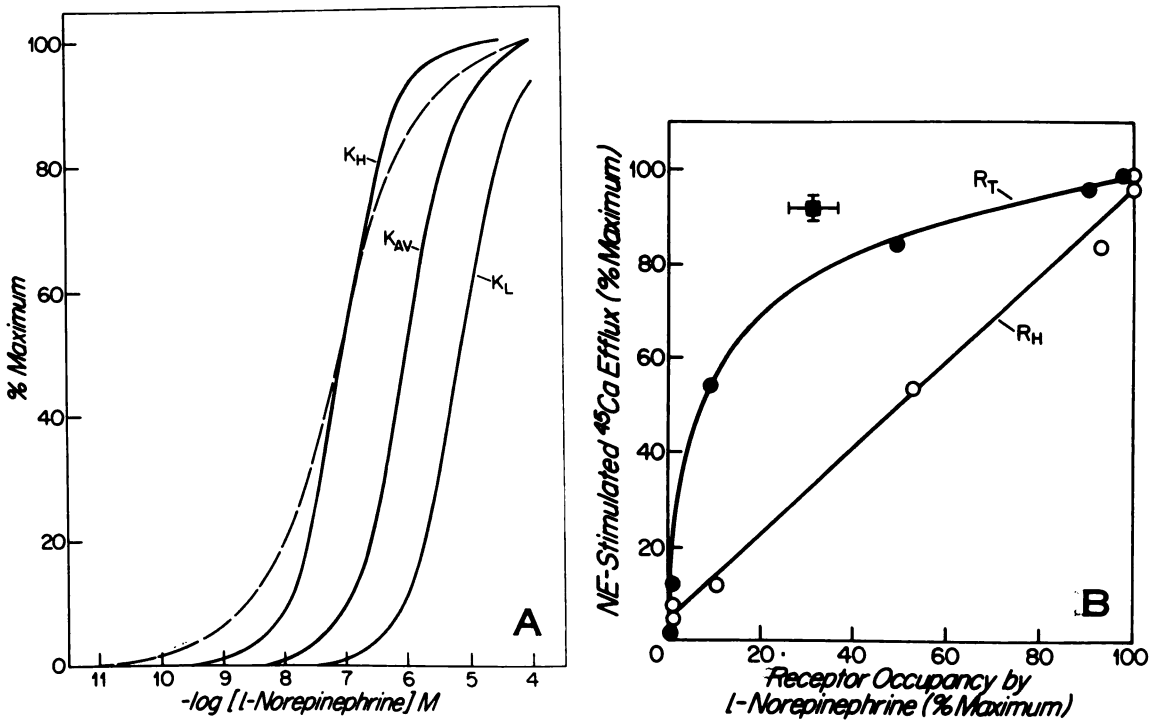

FIG. 4. *Receptor occupancy*

A, comparison of concentration-response curves for *l*-norepinephrine stimulation of ^{45}Ca efflux (- - -) and the occupancy of α_1-adrenergic receptors by *l*-norepinephrine (———), as determined by nonlinear curve fitting (LIGAND program) of the data in Fig. 2. K_{AV} depicts occupancy based on a one-site binding model for NE. K_H and K_L depict high and low affinity NE binding, respectively, based on a two-site model. High affinity NE binding (K_H = 92 nM) represented 43 ± 8% of the sites identified by [^{3}H]prazosin. B, the relationships between fractional occupancy of α_1-adrenergic receptors by *l*-norepinephrine and stimulation of ^{45}Ca efflux, based on analysis of NE binding to the receptor pool identified by [^{3}H]prazosin. The relationship between ^{45}Ca efflux and NE occupancy of all sites (●) is markedly nonlinear, whereas the relationship to only high affinity sites (○) is linear. The data are replotted from A. The results of the phenoxybenzamine inactivation experiment (■) are most consistent with the nonlinear relationship based on binding to all sites.

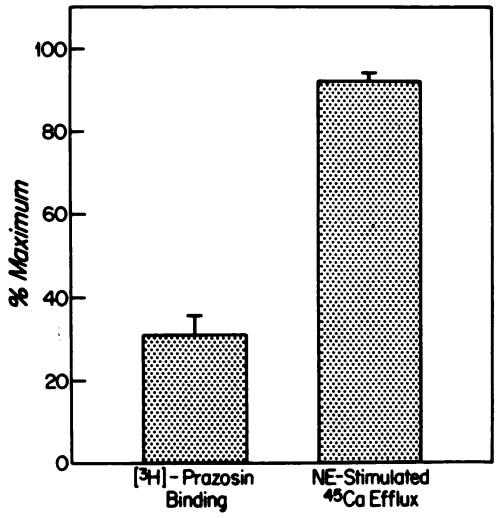

FIG. 5. *Dissimilar reductions in the density of [^{3}H]prazosin-binding sites and maximal norepinephrine-stimulated ^{45}Ca efflux in cells treated with phenoxybenzamine to irreversibly inactivate α-adrenergic receptors*

The data depicted represent the mean ± standard error for six experiments.

yohimbine[2] strongly suggests that cultured rabbit aortic smooth muscle cells contain few, if any, α_2 receptors.

[2] Unpublished observations.

This conclusion is in agreement with previous findings from contraction (18, 19) and calcium flux (19, 20) studies in intact rabbit aorta, and radioligand-binding studies in homogenized rabbit aorta (19, 21).

Scatchard analysis of [^{3}H]prazosin binding over an almost 100-fold concentration range was linear, and the prazosin competition curve was steep with a slope factor of unity, thus indicating that prazosin binds to all labeled receptors with a single affinity. Likewise, analysis of both [^{3}H]prazosin saturation and prazosin competition curves by nonlinear curve fitting was most consistent with a one-site binding model. In contrast, agonist competition curves for the [^{3}H]prazosin-binding site were shallow, with slope factors of less than 1. Nonlinear curve-fitting analysis of the NE competition curves, although consistent with the existence of two binding sites of different affinities for agonists, cannot be used to distinguish the possibility of discrete receptors from that of a single receptor with two affinity states. Nevertheless, these data indicate that, in this system, agonists bind in a complex manner that is fundamentally different from antagonists.

Similar methods of NE competition curve analysis have been used to demonstrate both high and low affinity agonist-binding states of α_1-adrenergic receptors in particulate fractions of rat liver (22), kidney (23), and myocardium (24). In each instance, the high affinity binding

state is regulated by guanine nucleotides and cations. Our observations showing no effect of guanine nucleotides on NE binding raise the possibility that coupling of α_1-adrenergic receptors in rabbit aorta does not involve a guanine nucleotide-sensitive site, or alternately, that the coupling relationship in these broken cells differs from *in vivo* conditions sufficiently to make a guanine nucleotide effect difficult to detect. Interestingly, α_1-adrenergic receptors in two other cultured cell systems also appear to be guanine nucleotide insensitive (25, 26).

Using the iodinated radioligand [^{125}I]BE-2254, Wikberg *et al.* (3) previously characterized α_1-adrenergic receptors in vascular smooth muscle cells cultured from the rabbit aorta. The characteristics of the α_1-adrenergic receptor identified by [^{3}H]prazosin in our study differ from those reported by Wikberg *et al.* in that the binding affinities for prazosin and NE are higher in our study, as is the ratio of prazosin to yohimbine-binding affinities.

NE-stimulated ^{45}Ca efflux. An increase in the concentration of intracellular ionized calcium is thought to be a key step in excitation-contraction coupling in vascular smooth muscle (27, 28). It has been shown that in rabbit aorta α_1-adrenergic agonists mobilize intracellular calcium as well as increase the influx of extracellular calcium (19, 20, 28). The initial phase of NE-induced vascular smooth muscle contraction occurs in the absence of extracellular calcium or in the presence of lanthanum to block calcium influx, whereas the tonic phase of NE-induced contraction is abolished by removal of extracellular calcium (28). Since in cultured rabbit aortic smooth muscle cells NE-induced ^{45}Ca efflux is largely unaffected by removal of extracellular calcium from the buffer,[2] it appears that mobilization of calcium from intracellular storage sites plays a major role during α-adrenergic receptor activation. The high sensitivity and rapid time course of NE-stimulated ^{45}Ca efflux in cultured rabbit aortic smooth muscle cells are consistent with the contractile effects of NE in intact rabbit aorta (8, 9, 18, 19, 29). Likewise, prazosin caused a concentration-related decrease in NE-stimulated ^{45}Ca efflux (maximal inhibition occurred at 100 nM), thus indicating that NE-stimulated ^{45}Ca efflux in this cell system is mediated entirely by adrenergic receptors of the α_1 subtype.

Relationship between fractional receptor occupancy and ^{45}Ca efflux. The major finding of this study is that the relationship between receptor occupancy by NE and NE-stimulated ^{45}Ca efflux is markedly nonlinear, such that 50% of maximal NE-stimulated efflux occurs when only approximately 7% of total receptors are occupied. The nonlinearity of this relationship was confirmed by a second method in which 69% of receptors were irreversibly inactivated by phenoxybenzamine pretreatment of intact cells. Under these conditions, maximal NE-stimulated ^{45}Ca efflux was only minimally decreased by 8%, further indicating that a near-maximum cellular response to α-adrenergic stimulation requires only a small percentage of the total receptors present.

In both vascular and nonvascular tissues, it has been observed that the maximum response to a variety of stimulatory hormones can be achieved by occupying only a small fraction of total hormone receptors, a phenomenon most often attributed to the presence of spare receptors. This concept, proposed by Stephenson (30), Nickerson (31), and Furchgott (32), and recently reviewed by Ruffolo (5), states that, in the presence of a receptor number exceeding that necessary for a maximum tissue response, a reduction in total receptor number initially causes a rightward shift in the concentration-response curve without a reduction in maximum response until all excess (spare) receptors are inactivated, at which point a further reduction in receptor number results in a reduction in maximum response.

In most prior investigations, the α-adrenergic receptor occupancy-response relationship has been determined inferentially, based on the shift in the concentration-response relationship caused by various degrees of receptor inactivation by phenoxybenzamine (5, 8, 9, 32). More recently, due to the availability of α-adrenergic receptor-specific radioligands, it has been possible to determine receptor occupancy directly, and therefore to test the spare receptor hypothesis. By comparing the contractile response to α-adrenergic agonists in rat vas deferens with α-adrenergic receptor occupancy determined by radioligand binding in homogenates of the same tissue, Minneman *et al.* (10) observed a markedly nonlinear occupancy-response relationship. These investigators further noted that progressive receptor inactivation by phenoxybenzamine caused the predicted rightward shift in the apparent dissociation constant, thereby supporting the presence of spare receptors (11). However, they also found that a 39% reduction in receptor number by phenoxybenzamine resulted in an equivalent decrease in maximum contractile response, an observation at odds with the spare receptor hypothesis and more consistent with a linear occupancy-response relationship (11). Only one previous study has directly examined the α-adrenergic receptor occupancy-response in a vascular tissue. Sastre *et al.* (12) also observed a markedly nonlinear relationship between receptor occupancy determined by radioligand binding to tissue homogenates and contractile response in canine aorta. However, a maximal contractile response did not occur until 97% of receptors were occupied; therefore, it was concluded that spare receptors most likely were not present.

Inspection of the occupancy-response relationship determined by NE competition for the [^{3}H]prazosin-binding site (Fig. 4B) indicated that, in cultured rabbit aortic smooth muscle cells as in the canine aorta, a maximal cellular response requires occupancy of essentially all receptors. However, a more direct assessment of this issue by means of phenoxybenzamine receptor inactivation indicates that near maximal NE-stimulated ^{45}Ca efflux can be elicited after inactivation of 69% of receptors (Figs. 4B and 5). Since it is now known that α-adrenergic receptor affinity for agonists can be influenced by several factors including cellular homogenization, temperature, guanine nucleotides, and cations (24, 33), it is possible that estimation of receptor occupancy based on agonist-binding curves in homogenized tissues may not reflect accurately the occupancy-response relationship in intact cells. By contrast, the effects of phenoxybenzamine inactivation on ^{45}Ca efflux would be

unaffected by alterations in receptor affinity induced by the assay conditions or homogenization, and thus are more likely to reflect the occupancy-response relationship in intact cells accurately. Based on these considerations, the present data are most consistent with the conclusion that the majority of α-adrenergic receptors identified by [^{3}H]prazosin in cultured rabbit aortic smooth muscle cells are not required for maximum NE-stimulated ^{45}Ca efflux, and therefore may be considered spare receptors.

An alternative view of the nonlinear occupancy-response relationship, as proposed by Sastre *et al.* (12), would involve a postreceptor amplification step. Although the phenoxybenzamine inactivation data demonstrate that near-maximum calcium efflux can be induced by occupancy of only a fraction of the available receptors, the locus of this nonlinearity cannot be determined from the present experiments. Since calcium flux, a step proximal to contraction, was evaluated, these data indicate that nonlinearity of the α-adrenergic receptor occupancy-response relationship occurs, at least in part, at a step proximal to calcium mobilization. It will be important to determine whether α-adrenergic receptor-coupled steps proximal to intracellular calcium release [e.g., inositol 1,4,5-trisphosphate production (34)] are linearly or nonlinearly related to receptor occupancy.

The binding of NE to α-adrenergic receptors in cultured rabbit aortic smooth muscle cell homogenates was best described by a two-site model in which 43% of sites were of high affinity. Interestingly, the relationship between occupation of only these sites and ^{45}Ca efflux was highly linear (Fig. 4B), thus raising the possibility that high affinity binding reflects receptors in the coupled state. This possibility, although attractive, cannot be concluded from these data, since binding experiments were performed in cellular homogenates which may not necessarily reflect the actual proportions of high and low affinity sites present in intact cells (33). The significance of two agonist-binding affinities under these experimental conditions is not known, but potentially may reflect functional heterogeneity of vascular α-adrenergic receptors.

Cultured vascular smooth muscle cells offer a number of advantages for the study of α-adrenergic receptor mechanisms at the cellular level. Because these cells are derived entirely from the medial layer of the rabbit aorta, a location which is known to consist entirely of vascular smooth muscle cells, there is no contamination with other vascular or nonvascular cell types. The ability to manipulate the cellular environment and maintain precisely controlled experimental conditions for relatively prolonged periods of time allows assessment of the influence of the cellular milieu on adrenergic receptor expression and function. Finally, because of the absence of tissue diffusional barriers, the cultured vascular smooth muscle cell offers unique opportunities for evaluating receptor-coupled ionic fluxes.

In summary, vascular smooth muscle cells cultured from the rabbit aorta express α_1-adrenergic receptors which are coupled to cellular calcium flux. The NE occupancy-response relationship for the total receptor pool is markedly nonlinear, and following irreversible inactivation of 69% of receptors by phenoxybenzamine, the magnitude of maximal NE-stimulated ^{45}Ca efflux is only minimally reduced. These data are most consistent with the presence of a sizable pool of spare α_1-adrenergic receptors. Approximately 40% of receptors exhibit high binding affinity for NE and demonstrate a linear occupancy-response relationship with ^{45}Ca efflux, thus raising the possibility that heterogeneity of agonist binding may reflect differences in the state of receptor coupling. These findings have important implications for the analysis of radioligand-binding studies of α-adrenergic receptors and the understanding of vascular α-adrenergic responsiveness.

ACKNOWLEDGMENTS

The authors would like to acknowledge the expert technical assistance of Gwendolyn M. Wise in the performance of these experiments, and of Anne F. Brock and William J. Atkinson in the culturing of rabbit aortic smooth muscle cells.

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Send reprint requests to: Wilson S. Colucci, M.D., Cardiovascular Division, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.