Characterization of Postsynaptic Alpha-Adrenergic Receptors by \(^{[3]H}\)-Dihydroergocryptine Binding in Muscular Arteries from the Rat Mesentery

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SUMMARY Alpha-adrenergic receptors are likely to be important determinants of the effects of catecholamines on vascular resistance. To study the \(\alpha\)-adrenergic receptor in muscular arteries of the type that determine vascular resistance, we characterized and quantitated \(\alpha\)-adrenergic receptors in a particulate fraction of the highly reactive, richly innervated arteries of the rat mesentery. With the ligand \(^{[3]H}\)-dihydroergocryptine (\(^{[3]H}\)-DHEC), specific binding (displaceable by 5 \(\mu\)M phentolamine) is saturable. There is a single class of binding sites with a dissociation constant \((K_d)\) of 2.9 nM and a maximal binding capacity of 68 fmoles of \(^{[3]H}\)-DHEC per mg of particulate fraction protein. Catecholamines compete for \(^{[3]H}\)-DHEC binding stereospecifically and with the \(\alpha\)-adrenergic potency series of \((-\beta\)-epinephrine > \((-\beta\)-norepinephrine > \((-\beta\)-isoproterenol. Binding is rapid \((t_{1/2} \leq 2\) mins) and rapidly reversible \((t_{1/2} \leq 2\) mins). Inhibition of \(^{[3]H}\)-DHEC binding by the \(\alpha\)-adrenergic antagonist phentolamine \((K_d = 8200\) nM) is much greater than by the \(\beta\)-adrenergic antagonist propranolol \((K_d = 63\) nM) is 20 times more potent in competing for \(^{[3]H}\)-DHEC binding than is the \(\alpha\beta\)-selective antagonist yohimbine \((K_d = 1250\) nM), thus suggesting that the \(\alpha\)-adrenergic receptor identified is predominantly of the \(\alpha\)-subtype that is responsible for vascular smooth muscle contraction. This extension of radioligand binding techniques to highly innervated muscular arteries of the type contributing to vascular resistance will allow the study of the role of the vascular \(\alpha\)-adrenergic receptor in various physiologic states and models of hypertension.

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KEY WORDS \(\alpha\)-adrenergic receptors \(\cdot\) spontaneously hypertensive rat \(\cdot\) mesenteric artery \(\cdot\) \(^{[3]H}\)-dihydroergocryptine

THE sympathetic nervous system directly influences vascular resistance through the interaction of catecholamines with specific receptors associated with the vascular smooth muscle cells of muscular arteries and arterioles. In blood vessels, catecholamine-induced constriction is mediated by \(\alpha\)-adrenergic receptors. In recent years, important advances have been made in the understanding of adrenergic receptors through the use of radioactive ligands that bind selectively to these receptors, thus permitting their direct characterization. This experimental approach has revealed that adrenergic receptors are distinct molecular entities subject to physiologic regulation and pathologic alteration, and thus, are potentially important loci of biologic control. Radioligand binding assays have been used to study adrenergic receptors in a great number of target tissues and cells in culture. The role of adrenergic receptors in modulating the variable vascular responsiveness to catecholamines observed under different physiologic and pathophysiologic conditions, however, has not been investigated directly with these techniques.

Two technical limitations have hindered progress in the definition of the regulatory role of the vascular \(\alpha\)-adrenergic receptor. First, radioactive ligands of high specificity and affinity for \(\alpha\)-adrenergic receptors and of suitable specific activity for the assay of relatively small amounts of tissue have only recently become available. Second, since arterial walls are structurally complex, and contain connective tissue, neural elements, and endothelial cells in addition to smooth muscle, it has been difficult to obtain fractions of vessel walls sufficiently enriched in smooth muscle to permit assay of adrenergic receptors. This difficulty in obtaining an adequate amount of tissue for ligand binding assays is reflected in the fact that all direct...
studies of vascular receptors that have been reported to date have been performed on aortic membranes.  

There is a single report of the successful identification of vascular α-adrenergic receptors in a homogenate of canine aorta with the ligand [3H]-dihydropregynephrine (3H)-DHEC.  

It has been stressed, however, that blood vessels from various parts of the circulation exhibit considerable histologic, physiologic, and pharmacologic heterogeneity. Thus, this approach of using a large, fibroelastic, conduit-type vessel may not be applicable to the study of adrenergic receptor physiology in highly innervated, reactive muscular arteries of the type that contributes to vascular resistance. In addition, there is a need for such studies in a small laboratory animal such as the rat, for it can be readily subjected to hormonal manipulation and has been genetically inbred to produce a model of hypertension that appears to have a neurogenic component.

As an initial approach to the study of the physiology and biochemistry of adrenergic receptors in muscular arteries, we have identified [3H]-DHEC binding sites that have the characteristics of α-adrenergic receptors in a particulate fraction from rat mesenteric arteries. Data obtained through the use of subtype-selective antagonists suggest that these receptors are predominantly of the α-subtype mediating vascular smooth muscle contraction.

Methods

Materials

We obtained [3H]-DHEC from New England Nuclear (Boston, MA). It had a specific activity of 38.8 Ci/mmol and was greater than 98% pure on thin-layer chromatography in two solvent systems (chloroform:ethanol:acetic acid, 9:5:1, and chloroform:benzene:ethanol:ammonium hydroxide, 4:2:1:0.1). We stored [3H]-DHEC in the dark in distilled water in a test tube wrapped in aluminum foil.

Phentolamine-HCL was obtained from CIBA Pharmaceutical Co. (Summit, NJ); (+)epinephrine and (+)-norepinephrine from Sterling-Winthrop, (NY, NY); phenoxybenzamine from Smith, Kline and French Laboratories (Philadelphia, PA); (+)-propranolol from Ayerst Laboratories (New York, NY); clonidine-HCL from Boehringer Ingelheim Ltd. (Elmsford, NY); methoxamine from Burroughs Wellcome Co. (Research Triangle Park, NC); and prazosin from Pfizer Pharmaceuticals (New York, NY). Other compounds used were obtained from standard chemical suppliers.

Particulate Fraction Preparation

A particulate fraction of mesenteric artery smooth muscle was prepared by modification of the methods described by Wei et al.  

Male Sprague-Dawley rats (300–350 g) (Charles River Laboratories, Wilmington, MA) were sacrificed by a blow to the head and cervical dislocation. The small intestine was doubly ligated at the proximal duodenum and terminal ileum, and cut between ligatures. The superior mesenteric vascular arcade was severed near its origin at the aorta and transferred, still attached to the small intestine, into ice-cold phosphate-buffered saline on the surface of a chilled Petri dish. The entire vascular arcade was then freed from its attachments to the bowel. The mesenteric vein, fat, and lymph nodes were removed from the artery by blunt dissection. Four arteries at a time were placed in an ice-cold 0.25 M sucrose solution (pH 6.1, unbuffered) in a Potter-Elvehjem homogenizer, and the fatty adventitial tissues removed with two gentle strokes of a specially ground, tapered Teflon pestle (clearance, 0.016 in.). Twelve arteries cleaned in this fashion were then combined in 40 ml of an ice-cold 0.25 M sucrose solution in a 50 ml beaker and coarsely minced with scissors. The tissue was transferred to a 125 ml Erlenmeyer flask, and another 20 ml of 0.25 M sucrose solution was added. The mix was then homogenized with a Brinkman Polytron (setting 8, 10 sec x 2) and poured into two 40 ml tubes that were centrifuged at 1500 x g for 10 minutes at 4°C. The supernatant was centrifuged at 100,000 x g for 30 minutes at 4°C in a Beckman ultracentrifuge. The supernatant was discarded, and any remaining fat on the sides of the tube was removed with a cotton-tipped applicator. The precipitate was resuspended in assay buffer (5 mM MgCl2, 50 mM Tris-HCl, pH 7.55) to a final protein concentration of 1.5–2.0 mg/ml. The mesenteric arteries from 12 300-g rats yielded approximately 3.8 mg of particulate fraction protein.

Radioligand Binding Assay

The [3H]-DHEC binding assay previously described for α-adrenergic receptors was modified to improve reproducibility when assaying the relatively small amount of protein available from rat mesenteric arteries. The assay mixture consisted of 25 μl [3H]-DHEC, 100 μl of the particulate fraction (0.15–0.20 mg protein), and 25 μl of distilled water or various agonists and antagonists dissolved in distilled water. The final [3H]-DHEC concentration under standard assay conditions was 3–5 nM. After incubation for 20 minutes at 22°C in a gyratory shaker bath, 125 μl aliquots were removed, diluted with 3 ml of assay buffer, and immediately filtered through a Whatman GF/C glass fiber filter. The filter was then washed four times with 5 ml portions of buffer. The filtration system consisted of a single 25 mm filter support (Department of Physiology Shop, Duke University, Durham, NC) mounted on a 1.0 liter filtration flask that was attached, through a flowmeter, to a vacuum line. The flow was adjusted so that 5 ml was filtered in 1–2 seconds. The flowmeter permitted the establishment of constant filtration conditions both within and between experiments. The use of this system permitted the reproducible generation of concentration response curves with a total of only about 200–300 cpm of specific binding. Specific binding was defined as that displacable by 5 μM phentolamine or 100 μM...
(-)epinephrine, and comprised about 40%-45% of the total bound counts at 3 nM [3H]-DHEC. All figures referred to specific binding. The filter "blank" retained about 0.1%-0.2% of the added counts.

Calculations

Saturation curves were analyzed by the method of Scatchard to determine the dissociation constant (Kd) and the amount of [3H]-DHEC bound. The Kd for nonradioactive agonists and antagonists was calculated by the method of Cheng and Prusoff with the formula Kd = IC50/[1 + (concentration of ligand/Kd ligand)], where IC50 = concentration of drug that inhibits [3H]-DHEC binding by 50%, and ligand = [3H]-DHEC. The IC50 of each agent was determined by logit analysis of dose response curves, each of which was the mean of two to four experiments performed in duplicate or triplicate. The Kd for [3H]-DHEC was also calculated from kinetic analysis as previously described.

Results

Saturability and Affinity of the [3H]-DHEC Binding Site

Specific binding was saturable in the presence of increasing concentrations of [3H]-DHEC (fig. 1). When

the saturation curves were analyzed by the method of Scatchard (fig. 1, insert), the plot of bound/free [3H]-DHEC versus bound [3H]-DHEC described a straight line (r = 0.92), suggesting that [3H]-DHEC binds to a single class of sites. The equilibrium dissociation constant (Kd) of [3H]-DHEC, represented by the negative reciprocal of the slope, was 2.9 nM. The maximum binding capacity of the site, represented by the x-axis intercept, was 68 fmoles of [3H]-DHEC per mg of particulate fraction protein.

Kinetics of Binding and Dissociation

Binding was rapid, reaching equilibrium in 6 minutes at 22°C, and remaining stable for at least 20 minutes (fig. 2). Since in these experiments the ligand concentration (3 nM) was much greater than receptor concentration (0.1 nM), pseudo-first-order reaction conditions were assumed. The observed forward rate constant (Kobs) was 0.809 min⁻¹ (fig. 2, insert), and the calculated second-order rate constant (Kc) was 6.53 × 10⁷ M⁻¹ min⁻¹. After equilibrium binding had been obtained, an excess of phentolamine (5 μM) was added to dissociate reversibly bound [3H]-DHEC. Dissociation occurred rapidly and was complete by 8 minutes (fig. 3). The first-order rate constant for the reverse reaction (k2) was 0.438 min⁻¹ (fig. 3, insert). The Kd calculated from the relationship Kd = k2/k1 was 6.7 nM.

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Specific binding of [3H]-DHEC to a particulate fraction from rat mesenteric artery in the presence of increasing concentrations of [3H]-DHEC. The points are mean values from two experiments performed in duplicate. Scatchard analysis of the binding data (insert) produced a straight line by the method of least squares (r = 0.92). The dissociation constant (Kd), determined as the reciprocal of the slope, equaled 2.9 nM. The maximum binding capacity, determined as the x-axis intercept, equaled 68 fmoles of [3H]-DHEC per mg of particulate fraction protein.
FIGURE 2. Time course of specific binding of \[^\text{[H]}\text{-DHEC}\] to a particulate fraction of rat mesenteric artery under standard assay conditions at 22°C. The data are the mean values of two separate experiments. Because the concentration of \[^\text{[H]}\text{-DHEC}\] was in marked excess (50-fold) of the concentration of receptor sites, the reaction may be considered pseudo-first order. Inset: The pseudo-first-order rate constant (\(k_{\text{obs}}\)), calculated by the plot depicted, was 0.809 min\(^{-1}\). \(B = \text{binding at each time, } B_{\text{max}} = \text{binding at equilibrium.}\)

FIGURE 3. Rate of dissociation of \[^\text{[H]}\text{-DHEC}\] from the particulate fraction of rat mesenteric artery at 22°C. After equilibrium binding was achieved, an excess of phentolamine (5 \(\mu\)M) was added to the reaction at time \(t = 0\). Dissociation was complete by 8 minutes. The data are the mean values of two separate experiments. Inset: The first-order rate constant (\(k_2\)), determined by the plot depicted, equaled 0.438 min\(^{-1}\). \(B = \text{binding at each time, } B_{\text{max}} = \text{binding at equilibrium.}\)

Specificity of [\(^\text{[H]}\text{-DHEC}\] Binding

Displacement of [\(^\text{[H]}\text{-DHEC}\] binding by catecholamine agonists followed the \(\alpha\)-adrenergic potency series of \((-)\text{epinephrine} > (-)\text{norepinephrine} > (-)\text{isoproterenol}\) (fig. 4). The (+)stereoisomers of epi-

nephrine and norepinephrine were less potent in competing with [\(^\text{[H]}\text{-DHEC}\] than were the \((-)\)isomers (fig. 4). The \(\alpha\)-adrenergic antagonist phentolamine (\(K_d = 3\) nM) was 2730 times more potent in competing for [\(^\text{[H]}\text{-DHEC}\] binding than was the \(\beta\)-adrenergic antagon-

ist (\(\pm\)propranolol (\(K_d = 8200\) nM) (fig. 5). Prazosin (\(K_d = 63\) nM), which is relatively specific for post-synaptic, or \(\alpha_1\) receptors, was 7 and 20 times more potent in displacing [\(^\text{[H]}\text{-DHEC}\] than were clonidine (\(K_d = 440\) nM) and yohimbine (\(K_d = 1250\) nM), which are thought to be relatively specific for presynaptic, or \(\alpha_2\) receptors.\(^{15,16}\) Dopamine and serotonin were approximately 100 times less potent in competing with [\(^\text{[H]}\text{-DHEC}\] than were \((-)\text{epinephrine}\) and \((-)\text{norepinephrine}. The catecholamine met-

abolite normetanephrine inhibited binding only at a high concentration (\(K_d = 100\) \(\mu\)M), while vanillylmandelic acid did not inhibit binding at 1 mM. The data for all compounds tested are summarized in table 1.

Discussion

The specific [\(^\text{[H]}\text{-DHEC}\] binding sites identified in this particulate fraction of rat mesenteric arteries had the characteristics of \(\alpha\)-adrenergic receptors. The binding was rapid, rapidly reversible, and saturable. Catecholamines competed for the [\(^\text{[H]}\text{-DHEC}\] bind-
FIGURE 4. Inhibition of $[^3H]$-DHEC binding by $\alpha$-adrenergic agonists. Each point represents the mean of duplicate or triplicate determinations from two to four experiments.

TABLE 1. Dissociation Constants ($K_d$) for Adrenergic Agonists, Antagonists, and Other Compounds

<table>
<thead>
<tr>
<th>Agonists and partial agonists</th>
<th>$K_d$ (10$^{-M}$) ± (SEM)$^{\dagger}$</th>
<th>Antagonists and other compounds</th>
<th>$K_d$ (10$^{-M}$) ± (SEM)$^{\dagger}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(−) Epinephrine</td>
<td>1.0 ± 0.2</td>
<td>Phentolamine</td>
<td>3 ± 0.3</td>
</tr>
<tr>
<td>(−) Norepinephrine</td>
<td>6.4 ± 0.2</td>
<td>Prazosin</td>
<td>63 ± 38</td>
</tr>
<tr>
<td>(+) Epinephrine</td>
<td>9.9 ± 1.9</td>
<td>Phenoxybenzamine</td>
<td>99 ± 45</td>
</tr>
<tr>
<td>(−) Isoproterenol</td>
<td>31 ± 3</td>
<td>Yohimbine</td>
<td>1,250 ± 200</td>
</tr>
<tr>
<td>(+) Norepinephrine</td>
<td>111 ± 14</td>
<td>(+) Propranolol</td>
<td>8,200 ± 300</td>
</tr>
<tr>
<td>Clonidine</td>
<td>0.44 ± 0.05</td>
<td>Normetanephrine</td>
<td>100,000</td>
</tr>
<tr>
<td>Methoxamine</td>
<td>10 ± 2</td>
<td>Serotonin</td>
<td>150,000</td>
</tr>
<tr>
<td>(+) Phenylephrine</td>
<td>15 ± 2</td>
<td>Catechol</td>
<td>NIT†</td>
</tr>
<tr>
<td>Dopamine</td>
<td>83 ± 55</td>
<td>Vanillylmandelic acid</td>
<td>NIT†</td>
</tr>
</tbody>
</table>

*Dissociation constants calculated by the method of Cheng and Prusoff, as described in Methods. Each value is the mean of duplicate or triplicate determinations from two to four separate experiments.

†SEM = standard error of the mean.

†Not inhibited at 1 mM.
ing sites in a stereospecific manner with the potency series of (-)epinephrine > (-)norepinephrine > (-)isoproterenol expected of α-adrenergic receptors. In addition, α-adrenergic antagonists were several-fold more potent in displacing [3H]-DHEC than were β-adrenergic antagonists, while high concentrations of catecholamine precursors and metabolites competed weakly or not at all. Ergot alkaloids, such as dihydroergocryptine, have complex pharmacologic properties and may be either antagonists or partial agonists for dopaminergic or serotoninergic as well as α-adrenergic responses. In bovine caudate nucleus, [3H]-DHEC labels dopaminergic receptors, and in rat cerebral cortex, possibly serotoninergic receptors as well as dopamine receptors. Pharmacologic experiments suggest that serotoninergic receptors are present in blood vessels; and dopaminergic vesiculoadrenergic responses have been described in mesenteric as well as renal arteries. It seems unlikely that [3H]-DHEC is binding, however, to any appreciable extent to either dopamine or serotonin receptors in the rat mesenteric artery particulate fraction. Both dopamine and serotonin were less than 1% as potent as (-)epinephrine in competing for [3H]-DHEC binding sites. Furthermore, the dopamine and serotonin displacement curves (not shown) were smooth and monophasic, thus providing no evidence for the presence of a second population of receptors for these agents. [3H]-DHEC also appears to label exclusively α-adrenergic receptors in canine aorta.

The rat mesenteric artery particulate fraction that we utilized has certain advantages in comparison to preparations of aortic membranes previously used for vascular receptor studies. Presumably, the aorta has been utilized in these studies because it can be readily dissected free of surrounding structures and because of its relatively large mass. In addition to smooth muscle cells, however, blood vessels contain endothelial cells, fibroblasts, varying amounts of connective tissue, and neural elements. Therefore, the likelihood of contamination of a particulate fraction by material from cell types in addition to smooth muscle appears to be greater with the use of fibroelastic as opposed to muscular type arteries, since the latter have a relatively high ratio of smooth muscle to total wall mass. The removal of the adventitia of the mesenteric arteries appears to greatly reduce contamination by neural elements. Evidence for this assertion derives from analysis of the binding data, discussed below. Because of its relatively small mass, the single layer of endothelial cells is unlikely to contribute importantly to the specific [3H]-DHEC binding of the mesenteric artery fraction. Thus, the density of α-adrenergic receptors in our preparation probably reflects accurately their density in vascular smooth muscle.

There are other advantages to studying vascular receptor mechanisms in muscular rather than fibroelastic arteries. First, since arteries from different vascular beds are pharmacologically heterogeneous, it is desirable to study mechanisms controlling vascular reactivity directly in muscular arteries that contribute to vascular resistance. Second, since functional innervation appears to be an important determinant of the number of postsynaptic adrenergic receptors, the densely innervated mesenteric arteries would appear to be a more appropriate model for studying physiologic regulation of vascular α-adrenergic receptors than the sparsely innervated aorta. Third, pathologic alteration of α-adrenergic receptor function may occur primarily in muscular arteries. Thus, the evidence for increased vascular α-adrenergic sensitivity in the spontaneously hypertensive rat is derived almost exclusively from muscular arteries. In contrast, the aorta of the spontaneously hypertensive rat has not been shown to have increased sensitivity to catecholamines.

Based on physiologic, pharmacologic, and direct biochemical evidence, α-adrenergic receptors may be divided into two subtypes termed α1 and α2. Alpha1 receptors are located postsynaptically and mediate classic α-adrenergic effects such as contraction of vascular smooth muscle. Alpha2 receptors are found primarily on presynaptic neurons where they inhibit norepinephrine release. Alpha2 receptors have been characterized on the human platelet and therefore are not exclusively presynaptic in location.

The α-adrenergic antagonists, prazosin and yohimbine, are subtype-selective for α1 and α2 receptors respectively; and, thus, are useful probes for identifying the relative proportion of these subtypes in various tissues. Because the ligand [3H]-DHEC labels both subtypes, the relative potency of prazosin and yohimbine in displacing bound [3H]-DHEC can provide an index of the proportion of α1 and α2 subtypes present in a given tissue. With this approach, it has been demonstrated that platelets contain predominantly α2; liver predominantly α1; and uterus both subtypes of α-adrenergic receptor.

In the rat mesenteric artery particulate fraction the predominant α-adrenergic receptor subtype appears to be α1. Although our method of analysis does not allow a precise quantitation of the proportion of each receptor subtype, there is considerable qualitative evidence to support this conclusion. Thus, prazosin is clearly more potent in displacing [3H]-DHEC (Kd = 63 nM) than is yohimbine (Kd = 1250 nM) in rat liver, a tissue thought to have 100% α1 subtype receptors, prazosin is also more potent than yohimbine in displacing [3H]-DHEC in platelets, a tissue thought to have 100% α1 subtype receptors, whereas in platelets, a tissue thought to have 100% α2 subtype receptors, the order of potency is reversed and yohimbine is 400 times more potent than prazosin. Prazosin is considerably more potent than yohimbine in displacing [3H]-DHEC from the rat mesenteric artery particulate fraction, suggesting that the predominant receptor subtype is similar to that in liver, α1. Supporting this conclusion is the observation that 10^-7 molar yohimbine, a concentration that completely inhibits [3H]-DHEC binding in the platelet, has no effect at all on binding in the rat mesenteric artery particulate fraction. Finally, the linear Scatchard plot is consistent with a single class
of receptors, although it has also been demonstrated that a linear plot may be obtained when the ligand used binds with equal affinity to both receptor subtypes.\textsuperscript{23} In summary, we have used [\(^3\text{H}\)]-DHEC to characterize and to quantify binding sites in rat mesenteric arteries that exhibit the characteristics of \(\alpha\)-adrenergic receptors. The \(\alpha\)-adrenergic receptors identified are predominantly of the \(\alpha_1\) subtype that mediates contraction of vascular smooth muscle. The ability to quantitate postsynaptic \(\alpha\)-adrenergic receptors in a highly reactive, muscular artery of the type that modulates vascular resistance will facilitate the study of the role of the \(\alpha\)-adrenergic receptor in various physiologic states and in animal models of hypertension.

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