Alpha-Adrenergic Receptors in Rat Myocardium
Identification by Binding of [3H]Dihydroergocryptine

R. Sanders Williams and Robert J. Lefkowitz

SUMMARY [3H]Dihydroergocryptine ([3H]DHE) binds to sites in membranes derived from rat myocardium that have the characteristics expected of α-adrenergic receptors. The binding is saturable with 41 fmol [3H]DHE bound per mg of protein and of high affinity with $K_D = 2.9$ nM. The binding is rapid and readily reversible. Adrenergic agonists compete with [3H]DHE for binding in the order: epinephrine $>$ norepinephrine $>$ isoproterenol; and adrenergic antagonists compete for binding in the order: phentolamine $>$ propranolol. For comparison, (−)[3H]dihydroalprenolol ([(−)[3H]DHA] was used to bind to sites in the same membrane preparations having characteristics of β-receptors. The number and affinity of β-receptors were quite similar to those of the α-receptors with 46 fmol (−)[3H]DHA per mg protein bound at saturation and $K_D = 2.5$ nM. These techniques allowed identification of both β- and α-adrenergic receptors in membranes derived from isolated atria, right ventricular free walls, and left ventricles including interventricular septa. This is the first report documenting direct identification of myocardial α-receptors by radioligand-binding techniques and complements the literature previously reporting myocardial inotropic and electrophysiological responses to α-adrenergic stimulation.

AHLQUIST’S original subdivision of responses to catecholamines into two distinct categories, α and β, has proven to be an extremely useful concept in the understanding of adrenergic hormones and drugs. Early work in this area seemed to indicate that the myocardial response to adrenergic stimulation was mediated exclusively through β-receptors. However, with the development of adrenergic agonists and antagonists having greater specificity for either the α- or the β-receptor, a large body of data has accumulated which suggests that the heart demonstrates responses mediated through α-receptors as well as β-receptors.

Several investigators have demonstrated that α-adrenergic agonists such as phenylephrine or the combination of epinephrine plus propranolol stimulate myocardial responses such as increased contractility, increased functional refractory period, increased duration of action potential, and decreased rate of phase 4 depolarization.$^{2-22}$ In addition, these responses are blocked by specific α-adrenergic antagonists, such as phentolamine, but not by β-adrenergic antagonists.

Radioligand binding studies have been used in the past few years to identify adrenergic receptors in numerous tissues, including the identification of β-receptors in rat and dog myocardium.$^{23, 24}$ The identification of binding sites in the myocardium having characteristics expected of α-receptors would provide further evidence for the existence of myocardial α-receptors, and this technique could be used to study adrenergic modulation of cardiac function at the molecular level.

The radioligand [3H]dihydroergocryptine ([3H]DHE, a potent α-adrenergic antagonist) has been used to identify binding sites that have the characteristics expected of α-adrenergic receptors$^{25, 26}$ in membrane fractions derived from smooth muscle-containing tissues such as rabbit uterus$^{27}$ and canine aorta,$^{28}$ as well as rat brain$^{29}$ and human platelets.$^{30}$ Accordingly, we undertook studies to determine if such radioligand-binding techniques could be used to document the existence of α-adrenergic receptors in the mammalian myocardium. In this communication we report the application of these methods to demonstrate and characterize α-adrenergic receptors in particulate fractions derived from rat myocardium.

Methods

Radioligands

[3H]DHE (specific activity, 25 Ci/mmol) was obtained from New England Nuclear and was shown to be homogeneous and indistinguishable from unlabeled dihydroergocryptine by thin layer chromatography in two different solvent systems.$^{27}$ Stored at $-20^\circ$C in ethanol and protected from light, it is stable for up to 6 months. Immediately prior to use, appropriate amounts of stock solution were diluted with deionized water containing 2.5 mM HCl and...
8% ethanol. This mixture was further diluted 6-fold by addition to the final incubation volume such that ethanol concentration in the final assay was 1%. Ethanol in final concentrations up to 4% has been shown to have no effect on specific binding of [3H]DHE.27 (-)[3H]DHA (specific activity, 33 Ci/mmmole) was also obtained from New England Nuclear and found to be homogeneous and identical to the parent compound by thin layer chromatography.31 Immediately prior to use, appropriate dilutions of stock solution stored in ethanol were made with deionized water.

Membrane Preparation

Male CD strain rats from Charles River weighing 150–350 g were killed by a blow to the head and the hearts rapidly excised and placed in buffer (4°C) consisting of sucrose, 0.25 M; Tris-HCl, 5 mM; MgCl₂, 1 mM; pH 7.4. Fat and great vessels were trimmed and removed. The hearts were then finely minced and homogenized in cold buffer with a Teflon pestle (Thomas) in a Potter-Elvehjem homogenizer driven at high speed. Pooled hearts from four to eight rats were used for each experiment.

Except where specified, both atria and both ventricles were used for the preparation of membranes. For the experiments in which receptors were studied in different anatomic regions of the heart, the hearts were placed in a shallow plastic dish filled with cold buffer and the atria separated from the ventricles by cutting along the atrioventricular sulcus. The interventricular septum and left ventricular free wall were then separated from the right ventricular free wall by grasping the septum with forceps and removing the right ventricular free wall as seen from above. The chambers were then kept separate and membranes prepared as stated.

The homogenates were filtered through a single layer of cheesecloth, then centrifuged at 1000 g for 10 minutes in a Sorvall RC-2B centrifuge. The pellets consisting of fibrous tissue and high density debris were discarded and the supernatant extracts were centrifuged at 40,000 g for 10 minutes. The resulting pellets were washed twice in cold incubation buffer consisting of sucrose, 0.25 M; Tris-HCl, 5 mM; MgCl₂, 1 mM; pH 7.4. Fat and great vessels were trimmed and removed. The hearts were then finely minced and homogenized in cold buffer with a Teflon pestle (Thomas) in a Potter-Elvehjem homogenizer driven at high speed. Pooled hearts from four to eight rats were used for each experiment.

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Binding Assay

Except where otherwise stated, membranes were incubated for 18 minutes at 25°C with [3H]DHE or (-)[3H]DHA in a total volume of 900 μl of incubation buffer. Incubations were ended by rapid vacuum filtration of the entire mixture through Whatman GFC glass fiber filters. The filters were immediately washed with 20 ml of incubation buffer (25°C) for [3H]DHE binding or 10 ml of incubation buffer (4°C) for (-)[3H]DHA. In separate experiments, these different wash volumes were shown to optimize the "specific" binding for each respective radioligand.

Filters were air dried overnight, placed in a Triton X-100/toluene-based fluid, and radioactivity was determined in a Packard liquid scintillation spectrometer at an efficiency of 50%. "Specific" binding of [3H]DHE was defined as that radioactivity bound to membranes which could be displaced by 10 μM phentolamine. "Specific" binding of [3H]DHE ranged from 35% to 60% of total counts bound per filter. However, if counts nonspecifically adsorbing to the filters (as calculated by pouring membrane-free incubation mixtures over filters and subjecting these to 20-ml washes identical to that used for membrane-containing incubation mixtures) are subtracted, the "specific" binding ranged from 50% to 75% of the total counts bound to the membranes themselves. For (-)[3H]DHA, "specific" binding was defined as that displaced by 10 μM (±)propranolol and ranged from 50% to 68%.

Counts bound to the filters in the absence of membranes (filter blank) ranged from 0.4% to 0.9% of the total counts filtered after the filters were washed in a manner identical to that used in the presence of membranes. In a typical experiment, total binding to membranes was 3.0% of total counts filtered, binding to membranes in the presence of 10⁻³ M phentolamine was 1.4% of total counts filtered, and specific binding was therefore 1.6% of total counts filtered or 53% of total counts bound. We call this "53% specific binding." If, however, a "filter blank" of 0.6% of total counts filtered is subtracted from total binding and from binding in the presence of 10⁻³ M phentolamine, thus estimating the binding to the membranes themselves, then the "specific binding to the membranes" could be calculated as (2.4 – 0.8)/2.4 = 67%. The presence or absence of phentolamine had no effect on the "filter blank."

In each experiment, binding was determined in triplicate identical incubation mixtures for each data point.

Results

Number and Affinity of Binding Sites; Kinetics of Binding

The binding of [3H]DHE to cardiac membrane fractions was found to be saturable and of high affinity (Fig. 1). Scatchard analysis of these data revealed a linear plot suggesting a single class of binding sites with 41 fmol of [3H]DHE bound per mg protein at saturation and a Kᵣ of 2.9 nm calculated from the negative reciprocal of the slope of the line (Fig. 1, inset).

Kinetic analysis of the binding of [3H]DHE to cardiac membranes showed it to be rapid with half-maximal binding by 2.5 minutes at 25°C. Binding remained at steady state for as long as 34 minutes (Fig. 2A). The binding of [3H]DHE was readily
reversed by the addition of $10^{-4}$ M phentolamine (Fig. 2B). We observed somewhat greater binding at equilibrium in the kinetic experiments as compared to the saturation experiments. In 10 saturation or competition experiments involving DHE at concentrations near 1.3 nM, the mean binding $\pm$ SEM was 14.6 $\pm$ 2.6 fmol/mg protein. In comparison, in five experiments examining kinetics at 1.3 nM DHE, the mean binding $\pm$ SEM was 20.0 $\pm$ 6.9 fmol/mg protein. This difference was not statistically significant.

In the experiments analyzing reversal of $[^{3}H]$DHE binding by the addition of $10^{-4}$ M phentolamine, we observed no decrement in binding at 30 and 60 seconds (Fig. 2B). We do not believe this represents the physiological reality, but suggest, rather, that the precision of the methods was not sufficient to detect small reductions in binding at these early points. A second possibility would be that inadequate mixing of the added phentolamine occurred (100 $\mu$l added to 7.1 ml incubation volume) despite gentle vortexing of the tubes.

**Specificity of Binding**

Adrenergic agonists competed with $[^{3}H]$DHE for the binding sites in the order expected of an $\alpha$-receptor, i.e., epinephrine > norepinephrine > isoproterenol (Fig. 3). Stereospecificity, another characteristic expected of adrenergic receptors, was demonstrated by the considerably greater potency...
Comparison of α- and β-Receptors

For comparison, binding of (−)[3H]DHA to cardiac membranes was measured using the identical membrane preparation and incubation conditions used to characterize the binding of [3H]DHE. (−)[3H]DHA has previously been shown to label sites indistinguishable from physiological β-receptors in both rat and dog cardiac membranes, though under experimental conditions somewhat different from those employed in the current studies.

The number of binding sites for (−)[3H]DHA was 46 fmol bound per mg of membrane protein, and a KD of 2.5 nM was calculated from the negative reciprocal of the slope of the Scatchard plot (Fig. 5). These values were quite similar to those deter-

TABLE 1 Inhibition of [3H]DHE Binding to Cardiac Membranes by Adrenergic Agents

<table>
<thead>
<tr>
<th>Agonists</th>
<th>EC50 (nM)</th>
<th>Potency relative to (−)epinephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>(−)Epinephrine</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>(−)Norepinephrine</td>
<td>2.5</td>
<td>0.080</td>
</tr>
<tr>
<td>(+)Epinephrine</td>
<td>15.0</td>
<td>0.013</td>
</tr>
<tr>
<td>(−)Phenylnephrine</td>
<td>22.0</td>
<td>0.009</td>
</tr>
<tr>
<td>(+)Norepinephrine</td>
<td>100.0</td>
<td>0.0020</td>
</tr>
<tr>
<td>(−)Isoproterenol</td>
<td>80.0</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antagonists and related compounds</th>
<th>Potency relative to (−)epinephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phentolamine</td>
<td>0.02</td>
</tr>
<tr>
<td>(±)Propranolol</td>
<td>48.0</td>
</tr>
<tr>
<td>Serotonin</td>
<td>75.0</td>
</tr>
<tr>
<td>Dopamine</td>
<td>170.0</td>
</tr>
</tbody>
</table>

Comparison of α- and β-Receptors

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minded for the sites binding \(^{3}H\)DHE in our membrane preparation.

The number and affinity of \(\beta\)-receptors in rat cardiac membranes as determined in this study differ somewhat from the values previously reported from this laboratory.\(^{23}\) While we have no definitive explanation for these differences, distinctly different experimental conditions (larger incubation volume, lower receptor concentration in the final membrane suspension, lower temperature) were employed and probably account for the difference in results.

It should be noted that high concentrations of \((-\)^{3}H\)DHA (greater than 15 \(n\)M) appeared to label a second order of binding sites with a much lower affinity. However, the accuracy of data obtained at these very high concentrations was poor (very low percentage of "specific binding"), and this low affinity site did not demonstrate the characteristics, such as stereospecificity, expected of \(\beta\)-adrenergic receptors. Experiments not shown here did confirm that \((-\)^{3}H\)DHA binding to the high affinity site demonstrated characteristics expected of \(\beta\)-receptors, i.e., stereospecificity. For these reasons, only points on the saturation curve obtained at \((-\)^{3}H\)DHA concentrations less than 15 \(n\)M were used in the Scatchard analysis quantitating number of binding sites and \(K_D\).

We do not have a clear explanation as to why the current experimental methods seemed to detect this low affinity, propranolol-displaceable, but "nonspecific" site when this phenomenon was not observed in earlier experiments.\(^{23}\) However, this again presumably relates to differences in experimental techniques cited above.

Anatomic Localization of \(\alpha\)- and \(\beta\)-Adrenergic Receptor Binding Sites

Using \(^{3}H\)DHE and \((-\)^{3}H\)DHA, we sought to determine the relative densities of the two classes of binding sites in several regions of the heart including the atria, the right ventricular free wall, and the left ventricular wall. In these studies the interventricular septum was included as part of the left ventricle.

The results indicate that both classes of binding sites are demonstrable in each region of the heart. In three experiments each involving pooled hearts from 8–20 rats, no significant difference was observed between the left and right ventricles in the binding of \(^{3}H\)DHE (Table 2). In two further experiments measuring the binding of \((-\)^{3}H\)DHA, membranes of left ventricular origin demonstrated a somewhat greater number of binding sites than those derived from right ventricles (Table 2). No differences in \(K_D\) were observed for either class of binding sites.

While specific binding to atrial membranes was observed for both \((-\)^{3}H\)DHA and \(^{3}H\)DHE, the small quantity of membrane protein available despite the use of up to 20 rats did not allow accurate quantification of \(K_D\) or total number of sites (data not shown).

### Discussion

#### Validation of the Existence of Myocardial \(\alpha\)-Receptors

\(^{3}H\)DHE was used in these experiments to identify binding sites in a particulate membrane suspension derived from rat myocardium that have the characteristics expected of \(\alpha\)-adrenergic receptors. The binding is of high affinity, saturable, stereospecific, and demonstrates forward and reverse kinetics appropriate for the binding of radioligand to adrenergic receptors. In addition, the \(\alpha\)-adrenergic agonists epinephrine and norepinephrine were 40 to 400-fold more potent in competing for the binding of \(^{3}H\)DHE than the \(\beta\)-specific agonist, isoproterenol, and the \(\alpha\)-adrenergic antagonist phentolamine was 2500-fold more potent than the \(\beta\)-specific antagonist, propranolol. These characteristics clearly distinguish these binding sites from those sites identified in myocardial membrane fractions by \((-\)^{3}H\)DHA which appear to represent \(\beta\)-adrenergic receptors.\(^{23,24}\)

While we do not report new physiological or biochemical responses correlating with the characteristics of radioligand binding, numerous prior reports document physiological myocardial responses to drug combinations that specifically stimulate \(\alpha\)-adrenergic receptors.\(^{25-29}\) To equate these observa-
tions in broken cell preparations with the control mechanisms of physiological responses in vivo would be somewhat premature. However, the striking similarity between the characteristics of DHE interaction with cardiac membrane binding sites and the pharmacological characteristics of α-adrenergic receptors strongly supports the contention that DHE identifies α-receptors at the molecular level.

An increase in contractile force of the myocardium is the most consistently reported response to α-specific stimulation, although some investigators have reported a negative inotropic response. A different mechanism of action from the positive inotropic effects of β-adrenergic stimulation is suggested by several findings: (1) the time course of the development of the positive inotropic effect differs between α- and β-stimulation; (2) the electrophysiological sequelae of α-stimulation are an increased duration of the action potential, increased functional refractory period, and decreased automaticity; and (3) all of which are in opposite direction to the effects of β-stimulation; (3) most investigators report either no change or a reduction in cyclic AMP in response to α-specific stimulation.

Previous Attempts to Identify Myocardial α-Receptors by Radioligand Binding

Previous studies using [3H]phenoxybenzamine to label binding sites in myocardial preparations have been reported. The haloalkylamine phenoxybenzamine is widely known to be a nonspecific alkylating agent that binds covalently to a variety of membrane components other than the α-receptor, and the tritium-labeled compound is of such low specific activity (33 mCi/mmol) as compared to [3H]DHE (25 Ci/mmol) that it is extremely unlikely that binding sites for this radioligand represent the physiological α-receptor. No kinetic studies, saturation curves, or competition curves with adrenergic agents were reported.

A preliminary report of binding of [3H]DHE to cardiac membranes has been published. However, the data presented in this paper are difficult to evaluate in terms of specific labeling of α-receptors, since no experiments documenting appropriate kinetics, stereospecificity, or order of agonist competition were reported.

Physiological Significance of Myocardial α-Receptors

Baroreceptor reflexes occurring in vivo make clear separation of the primary myocardial responses to α-and β-adrenergic stimulation and blockade more difficult than in isolated heart preparations. In intact animals and in man, the myocardial β-adrenergic responses seem clearly dominant over any direct responses mediated by α-receptors when mixed agonists are employed. This phenomenon for years obscured the very existence of myocardial α-adrenergic responses.

However, the distinct possibility exists that the α-mediated myocardial responses described earlier become more important in certain circumstances, most notably in the presence of β-adrenergic blockade. In addition, reports that other factors, such as hypothyroidism, decreased rate of pacing, and increased calcium concentration, may potentiate the response to α-specific stimulation suggest that myocardial α-receptors may assume particular significance in certain disease states as well.

Potential Future Usefulness of Direct Radioligand Binding Techniques to Measure α-Adrenergic Receptors

Altered adrenergic control of myocardial function may be implicated in a variety of pathological conditions including congestive heart failure, hypertension, hypo- and hyperthyroidism, and sudden arrhythmic death. Radioligand binding techniques complement classical pharmacological studies by providing an assessment of the characteristics of receptor binding at the molecular level. Especially by providing for the simultaneous assessment of both β- and α-receptors, these methods may prove useful in assessing changes in receptor characteristics associated with various disease states.

On a more basic level, the possibility has been suggested that myocardial α- and β-receptors are interconvertible forms of the same macro-molecule. The capacity to assess directly both species of receptors after interventions known to influence the relative magnitudes of the respective physiological responses to α- and β-stimulation should be of use in resolving this provocative issue.

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