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Biochemical evidence for presynaptic and postsynaptic α-adrenoceptors in rat heart membranes: Positive homotropic cooperativity of presynaptic binding

([^{3}H]dihydroergocryptine binding/nerve endings/norepinephrine release/allosteric receptor)

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ABSTRACT **In crude rat cardiac membrane preparations, [^{3}H]dihydroergocryptine (^{3}H-DHE) appears to bind to two classes of sites with limited capacity, differing in their specificities and their affinities. The first class of binding sites interacts preferentially with the postsynaptic α-adrenoceptor blocker ARC 239, as can be expected for postsynaptic α-adrenoceptors. The binding of ^{3}H-DHE to these receptors follows the law of mass action, with a high affinity for ^{3}H-DHE ($K_{d\ 25°C}$ = 1.67 ± 0.37 nM). Postsynaptic saturating levels of ^{3}H-DHE are necessary to occupy the second class of binding sites. These sites exhibit a preferential affinity for presynaptic ligands such as clonidine and yohimbine, as would be expected for presynaptic α-adrenergic receptors. This presynaptic binding shows a markedly positive homotropic cooperativity (Hill n = 2.88) with initial and final apparent K_ds of 23 and 0.83 nM, respectively. Free energy of interaction between sites is of the order of 2 kcal (8.36 kJ)/mol of sites. These characteristics provide a rational molecular basis for the functional role of presynaptic α-adrenoceptors that mediate the inhibition of norepinephrine release from nerve endings.**

In the peripheral sympathetic nervous system, norepinephrine released by nerve stimulation can interact with α- and β-adrenoceptors located on target cells and nerve endings. The interaction of the neurotransmitter with the target cells initiates the biological effects whereas the interaction with nerve endings further regulates release of catecholamine. Presynaptic β-adrenoceptor stimulation, operating at a low concentration of norepinephrine, enhances transmitter release; conversely, presynaptic α-adrenoceptor stimulation, triggered by higher concentrations of norepinephrine, inhibits its release from nerve endings (for review, see ref. 1).

A recent publication (2) from our laboratory reported that α-adrenergic receptors demonstrated in rat brain membranes with the partial agonist [^{3}H]dihydroergocryptine (^{3}H-DHE) could be differentiated into two classes of receptors showing similarities with peripheral presynaptic and postsynaptic α-adrenoceptors. This distinction was made possible by labeling α-adrenoceptors with ^{3}H-DHE in the presence of various α-adrenergic antagonists known to interact preferentially with either presynaptic or postsynaptic receptors. We now report the use of a similar binding technique to show the presence of α-adrenoceptors in a rat heart membrane preparation with the properties of presynaptic and postsynaptic receptors whose existence had been suggested by various pharmacological experiments (1, 3). This study was facilitated by preliminary observations (not shown) that heart membranes, in contrast to brain membranes, do not possess dopaminergic and serotonergic binding sites, which are also able to interact with ^{3}H-DHE (4, 5). This finding allowed us to perform a detailed study of the molecular properties of presynaptic and postsynaptic binding sites.

Three important results were obtained in the present investigation. (*i*) Heart membranes contain an equal or higher amount of presynaptic than of postsynaptic α-adrenoceptors. (*ii*) Postsynaptic α-adrenoceptors show a high-affinity interaction with ^{3}H-DHE according to the law of mass action. (*iii*) Interaction of ^{3}H-DHE with presynaptic receptor sites exhibits the characteristics of a markedly positive homotropic cooperativity. This property provides an explanation, at the molecular level, for the mechanism regulating norepinephrine release from nerve endings.

MATERIAL AND METHODS

Experiments were performed on a crude membrane preparation of rat heart, in order to avoid the possible loss of α-receptors which might result from more extensive purification. Homogenates were prepared by differential centrifugation between 400 and 28,000 × g according to the original method of Williams and Lefkowitz (6). The incubation buffer used for binding studies was 10 mM $MgCl_2$/50 mM Tris·HCl, pH 7.5. ^{3}H-DHE (New England Nuclear; specific activity, 24.1 Ci/mmol) (0.5–30 nM) and myocardial membranes (400–600 mg) were incubated in glass tubes with and without a high concentration (50 μM) of the unlabeled α-blocker phentolamine, in a final volume of 300 μl of incubation buffer. Incubations were stopped by diluting 200-μl aliquots with 4 ml of cold (4°C) buffer followed by rapid filtration through Whatman GF/C glass fiber filters. Filters were rapidly washed with 15 ml of cold incubation buffer, dried, and assayed for radioactivity in 6 ml of Permafluor solution in a Packard Prias PL Tri-Carb liquid scintillation counter with an efficiency of 42%. Specific binding was taken as the difference between radioactivity measured on the filters in the absence and presence of phentolamine and represented 50–60% of the total binding of ^{3}H-DHE.

Addition to the incubation mixture of saturating concentrations of compounds, such as prazosin or ARC 239, which preferentially interact with postsynaptic receptors (A. M. Huchet, P. Mouille, R. Kadatz, and H. Schmitt, personal communication) allows the measurement of ^{3}H-DHE binding to presynaptic receptor sites; conversely, the addition of saturating concentrations of compounds, such as yohimbine, piperoxan, or clonidine, which preferentially interact with presynaptic receptors, permits ^{3}H-DHE to bind preferentially to postsynaptic receptors. In the present study, the postsynaptic and presynaptic blocking drugs used were ARC 239 [which is more specific than prazosin (A. M. Huchet *et al.*, personal communication)] and yohimbine, respectively, added in various concentrations to the incubation mixture concurrently with ^{3}H-

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Abbreviation: ^{3}H-DHE, [^{3}H]dihydroergocryptine.

DHE. Each radioligand concentration was precisely calculated by determination of the radioactivity in the incubation homogenate, and ^{3}H-DHE binding is expressed in femtomoles per milligram of membrane protein determined by the method of Lowry *et al.* (7).

The drugs used in this study were obtained from the following manufacturers: (−)-epinephrine HCl, (−)-norepinephrine HCl, (−)-phenylephrine HCl, (−)-isoproterenol (+)-bitartrate, and yohimbine HCl (Sigma); phentolamine HCl (Ciba-Geigy); prazosin HCl (Pfizer); 2-[2,4-(O-methoxyphenyl)piperazin-1-yl]ethyl-4,4-dimethyl-1,3-(2H,4H)isoquinolindione dihydrochloride (ARC 239) and clonidine (Boehringer Mannheim).

RESULTS

General Properties of ^{3}H-DHE Binding. Specific ^{3}H-DHE binding to myocardial membranes was linear up to a protein concentration of 2.5 mg/ml. It reached a steady state at 25°C after less than 10 min and was maintained up to 50 min with a ^{3}H-DHE concentration of 0.3 nM, suggesting insignificant inactivation of the ligand or loss of binding sites during incubation. Reversibility was demonstrated by the complete displacement, in 10 min, of ^{3}H-DHE (0.3 nM) by addition at equilibrium of phentolamine in large amounts (50 μM). Specificity was demonstrated by the observation that various α-agonists and antagonists could displace the ^{3}H-DHE specific binding whereas isoproterenol was ineffective. At 1 μM, epinephrine, norepinephrine, clonidine, phenylephrine, yohimbine, phentolamine, prazosin, and ARC 239 displaced at least 90% of the specific binding of 3 nM ^{3}H-DHE. The displacement of ^{3}H-DHE with increasing concentrations of all these drugs resulted in biphasic curves (not shown), suggesting an interaction with at least two classes of sites with different affinities.

Analysis of Concentration-Dependent Binding Curve of ^{3}H-DHE. Equilibrium binding was determined after 30 min of incubation with ^{3}H-DHE at 25°C. Specific ^{3}H-DHE binding to rat heart membranes was studied as a function of ^{3}H-DHE concentration between 0.1 and 20 nM. Fig. 1 shows a typical curve. It is clear from this figure that the ^{3}H-DHE binding curve has a biphasic shape. Complete saturation was obtained at 15 nM. The Scatchard plot gives a polyphasic curve, suggesting heterogeneity of sites (8). At low ^{3}H-DHE concentrations (<1 nM), the curve appears to be rectilinear. The second part is biphasic, an upward deviation being followed by a downward deviation from the horizontal axis. Whereas the downward deviation suggested a phenomenon of positive homotropic cooperativity, the upward deviation may be due to negative cooperativity or to site heterogeneity.

For further analysis, we studied the effect of preferential presynaptic or postsynaptic α-adrenoceptor ligands on the ^{3}H-DHE binding curve.

Effect of ARC 239 on ^{3}H-DHE Binding (Postsynaptic α-Adrenoceptors). ARC 239 is a potent α-adrenergic antagonist that interacts preferentially with postsynaptic receptors. ^{3}H-DHE binding studies were performed at three different constant ARC 239 concentrations. When the ARC 239 concentration increased, the ^{3}H-DHE binding curves became more and more sigmoidal (Fig. 2*A*). From the data in Fig. 2*A* and Scatchard analysis it appears that ARC 239 preferentially competes for the high-affinity ^{3}H-DHE binding: curves obtained from the various concentrations of ARC 239 (not shown here) showed straight lines all crossing the abscissa at the same point, as is the case for competitive inhibitors. Analysis of this representation allows us to determine the constants of the classical equation of competitive inhibition:

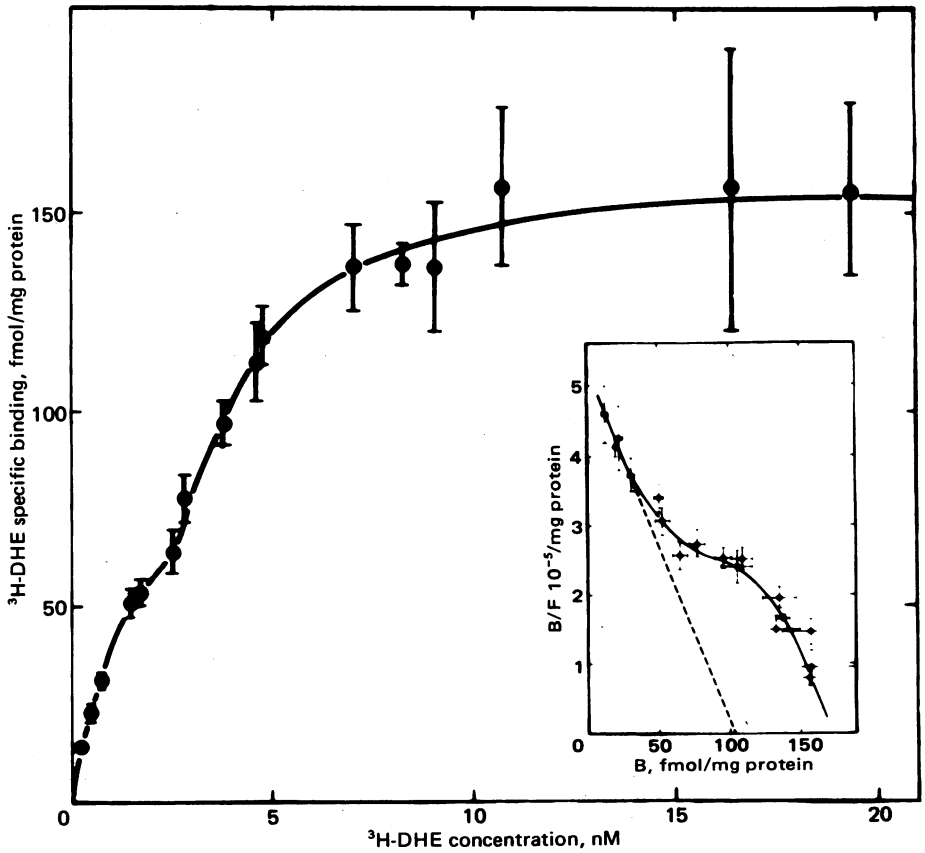

FIG. 1. ^{3}H-DHE concentration-dependent binding curve. Each experimental point was obtained in quadruplicate; the mean of these four values is given with the range of variations. (*Inset*) Scatchard plot.

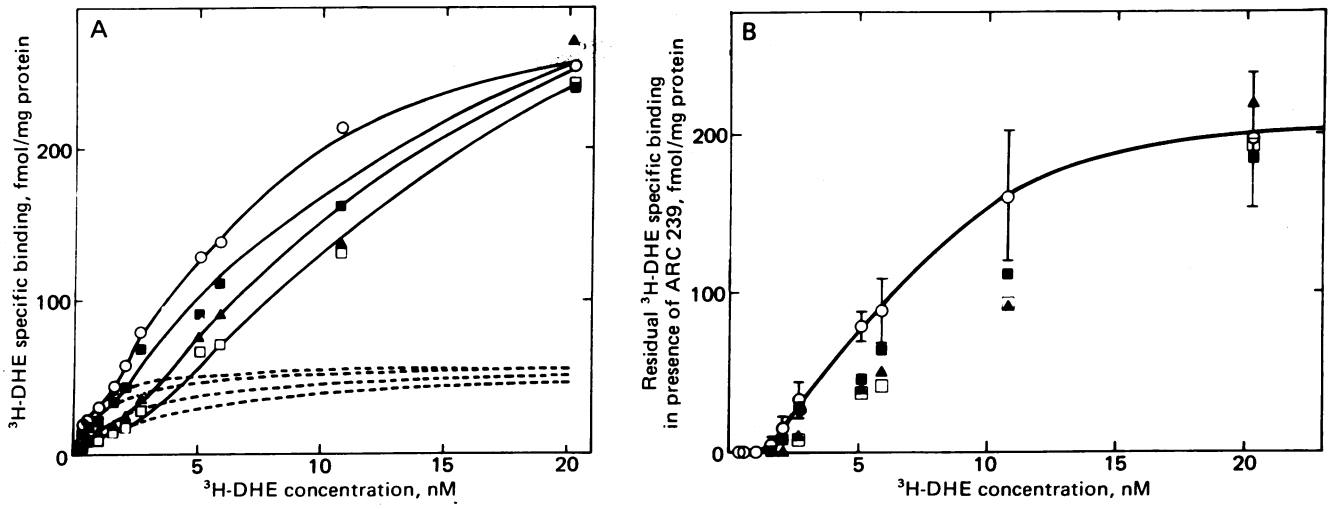

FIG. 2. (*A*) Effect of ARC 239 on the ^{3}H-DHE concentration-dependent binding curve. ○, No ARC 239; ■, 50 nM ARC 239; ▲, 20 nM; □, 40 nM. Broken lines calculated from Eq. 1. (*B*) Residual ^{3}H-DHE specific binding after subtraction of ^{3}H-DHE binding on postsynaptic α-adrenoceptors. The experimental points without ARC 239 (○) are given with their range of variation. The effects of various concentrations of ARC 239 are represented with the same symbols as in *A*.

$$\text{bound } ^3\text{H-DHE} = \frac{\text{maximal bound } ^3\text{H-DHE}}{1 + \frac{K_d}{[^3\text{H-DHE}]}\left(1 + \frac{[\text{ARC 239}]}{K_i}\right)} \quad [1]$$

in which K_d and K_i are the apparent dissociation constants of ^{3}H-DHE and ARC 239, respectively. This equation fits well the initial high-affinity ^{3}H-DHE binding which is competitively inhibited by ARC 239 (in Fig. 2*A* this high-affinity ^{3}H-DHE binding at each ARC 239 concentration is indicated by broken lines). The mean (±SEM) dissociation constant of ^{3}H-DHE obtained from seven experiments is 1.67 ± 0.37 nM and the number of high-affinity binding sites is 88 ± 15 fmol/mg of protein.

According to pharmacological experiments, the ARC 239-sensitive sites should correspond to the postsynaptic sites. Concentration-dependent binding curves of ^{3}H-DHE in the presence of various concentrations of ARC 239 were recalculated after subtraction of this postsynaptic component (residual ^{3}H-DHE binding). The curves obtained had a sigmoidal shape and, in contrast to the postsynaptic sites, did not show marked inhibition by ARC 239 (Fig. 2*B*). This analysis clearly shows the existence of a second class of sites that bind ^{3}H-DHE with a markedly homotropic positive cooperativity (see below). The number of these interacting sites obtained from seven experiments is 104 ± 24 fmol/mg of protein).

Effect of Yohimbine on ^{3}H-DHE Binding (Presynaptic α-Adrenoceptors). In order to investigate the nature of the residual sigmoidal ^{3}H-DHE binding, the effect of yohimbine, a preferential presynaptic antagonist, was studied. At ^{3}H-DHE concentrations close to sigmoidal binding saturation, the displacement of ^{3}H-DHE binding by increasing concentrations of yohimbine was studied. Fig. 3*A* shows a two-step inhibitory curve. The first step is inhibited by low yohimbine concentrations, but the second step requires much higher concentrations for inhibition. The addition of ARC 239 (0.7 μM) to the incubation mixture selectively inhibited the second step. This correlates well with the presynaptic nature of the first step which is well inhibited by low yohombine concentrations but not by ARC 239. The second step, ARC 239-sensitive and inhibited only by high concentrations of yohimbine, represents postsynaptic binding.

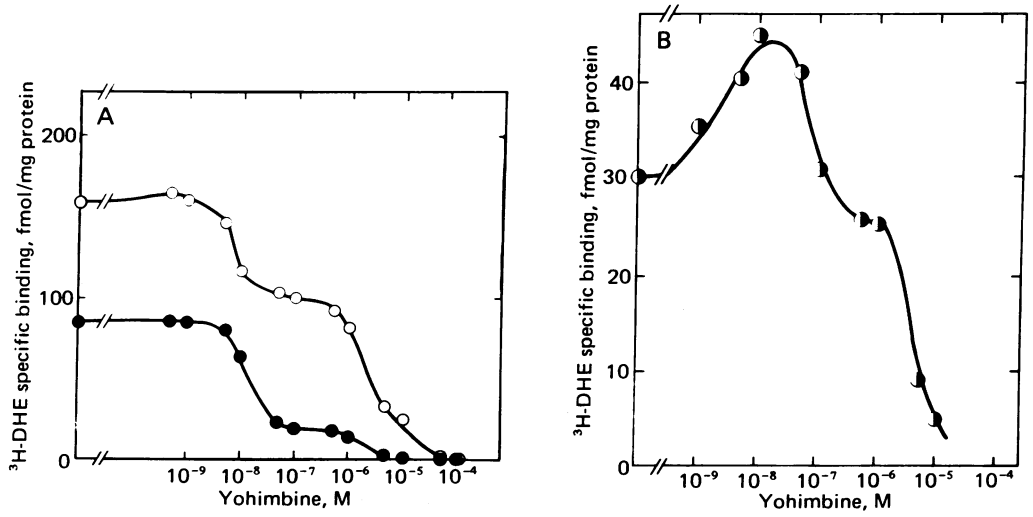

FIG. 3. Effect of yohimbine on specific ^{3}H-DHE binding. (*A*) At 9.4 nM ^{3}H-DHE. ○, No ARC 239; ●, 0.7 μM ARC 239. (*B*) At 2.7 nM ^{3}H-DHE in the presence of 20 nM ARC 239.

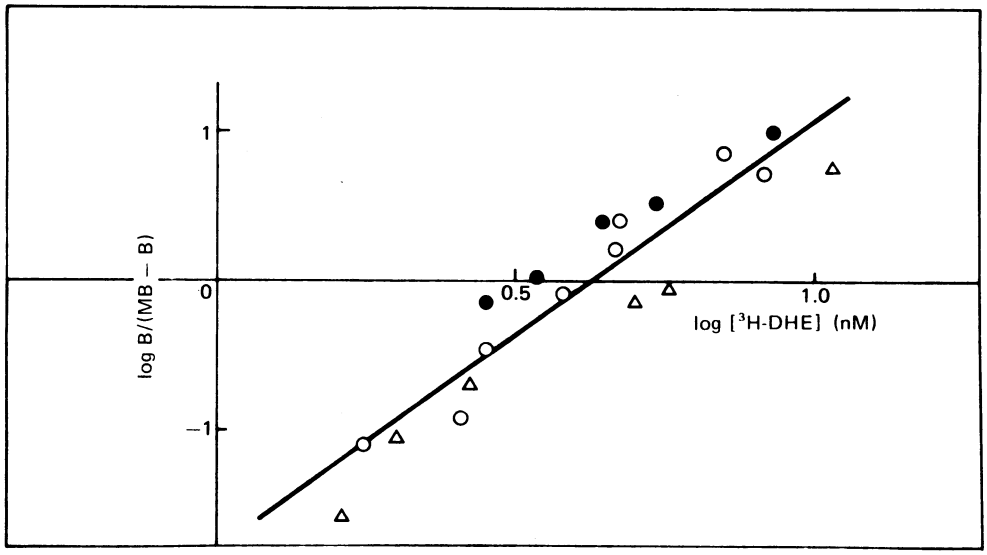

FIG. 4. Hill plot of ^{3}H-DHE presynaptic α-adrenergic binding. In this figure, the different symbols correspond to three different experiments. The straight line was obtained by linear regression analysis (r = 0.93; Hill n = 2.88). B, bound ^{3}H-DHE; MB, maximal bound ^{3}H-DHE.

The mutually exclusive effects of yohimbine and ARC 239 on ^{3}H-DHE binding show clearly that the residual sigmoidal binding shown in Fig. 2*B* is of presynaptic nature. At a ^{3}H-DHE concentration (2.7 nM) sufficiently low to occupy presynaptic receptors, an additional effect was observed. At low yohimbine concentrations, an increase in ^{3}H-DHE binding was observed (Fig. 3*B*). This experiment was performed in the presence of a low concentration of ARC 239 to partially block postsynaptic receptors which are almost saturated at the ^{3}H-DHE concentration used. Identical results have been obtained with clonidine, a preferential presynaptic agonist (1), as well as with epinephrine and norepinephrine. In contrast, postsynaptic ligands are unable to stimulate ^{3}H-DHE binding.

Allosteric Properties of Presynaptic α-Adrenergic Receptors. We have shown that rat heart membranes contain a significant amount of α-adrenoceptors having the properties of presynaptic α-adrenoceptors. In particular, the specific binding of ^{3}H-DHE by these receptors followed a sigmoidal curve as is the case for many allosteric proteins. In order to characterize the thermodynamic properties of this particular binding, we analyzed it using the phenomenological treatment described by Wyman (9, 10). This author demonstrated that a Hill plot representation of the binding data permits a simple calculation of the affinity change after site occupation by the ligand and of the free energy of interaction between sites. Thus, three different experiments similar to those of Fig. 2 were performed. The initial binding that follows the law of mass action was subtracted and then a Hill plot of the residual sigmoidal binding was prepared. The experimental error does not permit us to calculate accurately the Hill plot values of experimental points corresponding to a receptor occupancy smaller than 7% or greater than 92%. Therefore, the Hill curve of the experimental points was obtained within this occupancy range (Fig. 4). A linear regression analysis allowed us to draw a straight line (correlation coefficient, 0.93) that had a slope (Hill n) of 2.88. Using the thermodynamic analysis of Wyman, one may estimate that, between 7 and 92% receptor occupancy, there is a change in the apparent dissociation constant of 23 to 0.83 nM. Moreover, this affinity change corresponds to a minimum value of the free energy of interaction between sites of the order of 2 kcal/mol (1 kal = 4.18 kJ) of sites.

DISCUSSION

The analysis of the concentration-dependent binding curve of ^{3}H-DHE to rat heart membranes led to the hypothesis that two classes of binding sites were present. In a previous study (2) from our laboratory, we outlined a method which permits distinction between postsynaptic and presynaptic α-adrenoceptors. The former can be characterized as binding sites for ^{3}H-DHE interacting with high affinity with the postsynaptic blocking agents ARC 239 and prazosin; the latter are characterized as binding sites for ^{3}H-DHE interacting preferentially with a presynaptic agonist (clonidine) and antagonist (yohimbine). In rat heart membranes, the first class of ^{3}H-DHE binding sites is seen at concentrations of the tritiated ligand ranging from 0.1 to 2 nM. This binding is displaceable in a competitive manner by increasing concentrations of ARC 239. This compound is much less effective in displacing ^{3}H-DHE from the second class of binding sites. This observation suggests that the high-affinity binding sites for ^{3}H-DHE (K_d = 1.67 ± 0.37 nM) correspond to postsynaptic α-adrenoceptors. Analysis of the binding data indicates that binding of ^{3}H-DHE to these adrenoceptors proceeds according to the law of mass action.

The second class of ^{3}H-DHE binding sites interacts poorly with ARC 239 but shows marked interactions with yohimbine and clonidine. This suggests, therefore, that such sites correspond to presynaptic α-adrenoceptors. These receptors exhibit at least two categories of allosteric phenomena. First, there is a strong positive cooperativity between ^{3}H-DHE binding sites (Hill n = 2.88) with a minimal free energy of interaction between 7 and 92% site occupancy (KCAL/mol of sites); second, there is a dual effect of presynaptic inhibitors. Low doses of these inhibitors increase ^{3}H-DHE binding, and high doses are necessary to obtain ^{3}H-DHE binding inhibition. This is a classical allosteric effect of competitive inhibitors that at low concentrations act as allosteric activators (11). These results strongly suggest that presynaptic α-adrenoceptors are *allosteric receptors*. If this is the case, the thermodynamic analysis of Wyman (9, 10) allows us to formulate some predictions. Wyman demonstrated that, if intermolecular interactions between protein molecules are neglected, the Hill n provides a minimal value for the number of binding sites per allosteric molecule. In our

case we first may predict that presynaptic α-adrenoceptors contain at least three ^{3}H-DHE binding sites per receptor molecule. Second, the free energy of interaction is of the order of low-energy bonds such as those mediating allosteric interactions through conformational changes in the quaternary structure of oligomeric proteins (10, 12). Third, the ^{3}H-DHE final affinity for presynaptic α-adrenoceptors is very close to that of postsynaptic α-adrenoceptors. This raises the question of a possible structural analogy between isolated "subunits" of presynaptic α-adrenoceptors and the postsynaptic α-adrenoceptor, as is the case for the isolated chain of hemoglobin and myoglobin.

The biochemical recognition of postsynaptic and presynaptic adrenoceptors in rat heart is in agreement with previous pharmacological observations (3). Postsynaptic α-adrenoceptors are located in the membranes of myocardial or arteriolar cells, whereas presynaptic α-adrenoceptors are situated in the membranes of nerve endings (1). The presence of both classes of receptors in our preparation could be expected from the use of a crude membrane pellet which can contain both cardiac and nerve ending membranes. It would be interesting to examine the effects of 6-hydroxydopamine, in an attempt to destroy presynaptic nerve endings, on the yohimbine-sensitive DHE binding.

The present results may have important physiological and pharmacological implications. The high affinity and Michaelis–Menten kinetics of the postsynaptic α-adrenoceptors are in agreement with their function and location on postsynaptic cell membranes. The binding characteristics of the presynaptic receptors provide a satisfactory explanation for their functional properties assessed by pharmacological experiments. Because the role of presynaptic α-receptors is to trigger a reduction of norepinephrine release from nerve endings, it seems logical that they have an initial apparent affinity lower than the apparent affinity of postsynaptic receptors. This could explain their excitation only when sufficient significant levels of norepinephrine, to saturate the postsynaptic receptors, are present in the synaptic cleft. However, when these levels are reached, the efficiency of the negative feedback mediated by the presynaptic receptor is amplified by the positive cooperativity occurring at the level of binding.

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