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BINDING OF [^{3}H]-PRAZOSIN AND [^{3}H]-DIHYDROERGOCRYPTINE TO RAT CARDIAC α-ADRENOCEPTORS

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1 [^{3}H]-prazosin binds specifically to a single class of α-adrenoceptors in rat cardiac membranes (K_D25°C = 0.2 nM).

2 That these receptors are of the α_1-type was indicated by competition studies, i.e. α_1-antagonists such as prazosin and (2-(2, 6-dimethoxyphenoxyethyl) aminomethyl-1, 4-benzodioxane (WB 4101) were more potent than the α_2-antagonists, yohimbine and piperoxan in inhibiting [^{3}H]-prazosin binding.

3 A comparative study of [^{3}H]-prazosin binding and [^{3}H]-dihydroergocryptine binding to cardiac membranes showed that both [^{3}H]-prazosin and [^{3}H]-dihydroergocryptine (at low concentrations) bind to α_1-adrenoceptors, while [^{3}H]-dihydroergocryptine (at higher concentrations) also binds to another class of sites.

Introduction

The labelling of α-adrenoceptors was initially carried out with [^{3}H]-dihydroergocryptine. However, α-adrenoceptors have been subdivided into two classes (α_1 and α_2) according to their responses to different pharmacological agents (Langer, 1974). α_1-Adrenoceptors are postsynaptic receptors located on target cell membranes. α_2-Adrenoceptors may be present on nerve endings, where they regulate neurotransmitter release (Starke, 1972a, b) and also on certain non-neuronal membranes (Berthelsen & Pettinger, 1977).

Studies of the [^{3}H]-dihydroergocryptine binding have established the non-selectivity of this ligand, which binds to both α_1- and α_2-adrenoceptors in the central (Miach, Dausse & Meyer, 1978) and peripheral nervous system (Guicheney, Garay, Levy-Marchal & Meyer, 1978).

In order to confirm the existence of two classes of α-adrenoceptor binding sites in the rat heart, we have used [^{3}H]-prazosin, which has been shown to be selective for α-adrenoceptors in brain (Greengrass & Bremner, 1979; Miach, Dausse, Cardot & Meyer, 1980) and compared its binding characteristics to those of [^{3}H]-dihydroergocryptine.

Methods

Binding studies

Experiments were performed on a crude membrane preparation of male Wistar rat heart (200 to 250 g). Homogenates were prepared by differential centrifugation according to the original method of Williams, Mullikin & Lefkowitz (1976). The incubation buffer used for binding studies was 10 mM $MgCl_2$/50 mM Tris-HCl, pH 7.5. The radioligand, [^{3}H]-dihydroergocryptine or [^{3}H]-prazosin and myocardial membranes (1 mg/ml) were incubated at 25°C in glass tubes with and without a high concentration (10 μM) of the unlabelled α-adrenoceptor antagonist, phentolamine, in a final volume of 300 μl. Incubations were stopped by diluting 200 μl aliquots with 3 ml of cold (4°C) buffer followed by rapid filtration through Whatmann GF/C glass fibre filters. Filters were rapidly washed with 15 ml of cold incubation buffer, dried, and counted in 6 ml of Permafluor solution (Packard) 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. Each determination was performed in duplicate. [^{3}H]-dihydroergocryptine (sp. act.: 38.8 Ci/mmol) was purchased from New England Nuclear and [^{3}H]-prazosin (sp. act.: 33 Ci/mmol) was a gift from Pfizer. The radiochemical purity of [^{3}H]-prazosin was checked by ascending thin-layer chromatography on silica-gel plates using the solvent system ethyl acetate:methanol:diethylamine (R_F = 0.7) and was found to be 98%. No degradation was detectable after 40 min of incubation with myocardial membranes at 25°C. Each radioligand concentration was calculated after determination of the radioactivity in the incubation medium, and membrane protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

The drugs used in this study were obtained from

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the following manufacturers: (−)-adrenaline bitartrate, (+)-adrenaline bitartrate, (−)-noradrenaline bitartrate, (+)-noradrenaline bitartrate, (±)-erythro-α-methyl noradrenaline HCl (Sterling Winthrop); prazosin HCl (Pfizer); dihydroergocryptine mesylate (J. Logeais); yohimbine HCl, (−)-phenylephrine HCl, (±)-propranolol HCl, (±)-isoprenaline HCl, dopamine HCl (Sigma); piperoxan (Merck); clonidine (Boehringer Ingelheim); indoramin (Weyth); S3341 [(N-dicyclopropyl methyl) amino-2 oxazoline] (Servier); WB 4101 [(2-(2, 6-dimethoxyphenoxyethyl) aminomethyl-1, 4-benzodioxane] (Ward Blenkinsop & Co); phentolamine HCl (Ciba-Geigy).

6-Hydroxydopamine treatment

6-Hydroxydopamine hydrochloride (Fluka) was diluted to a concentration of 10 mg/ml in 0.9% w/v NaCl solution (saline) containing ascorbic acid (0.5 mg/ml). Young rats of either sex were treated for 3 weeks after birth with 7 subcutaneous injections of 6-hydroxydopamine at days 1, 3, 5, 8, 11, 15, 19 (100 mg/kg). Controls rats received only ascorbic acid/saline. Rats were decapitated 2 days after the last injection and 3 binding experiments were performed with 15 controls and 15 treated rats in each case.

Tissue noradrenaline determination

Rats were killed by decapitation and the left auricle together with part of the ventricle was rapidly removed, frozen in liquid nitrogen and homogenized in 0.4 M $HClO_4$ containing 10^{-5}M ascorbic acid in a glass-glass homogenizer, then ultrasonicated and centrifuged at 40,000 *g*. The supernatants were stored at −20°C for later noradrenaline determination. This was determined after separation by high pressure liquid chromatography (h.p.l.c.) using a column (25 × 0.2 cm i.d.) packed with a microparticulate octadecyl bound silica (Micropack CH 10 VARIAN) and 0.1 M sodium phosphate buffer pH 6.7 containing 1.43 mM sodium octyl sulphate as the mobile phase. The electrochemical detection of noradrenaline was performed at an applied potential of + 0.8V versus a Ag, AgCl reference electrode. The amperometric cell, with a carbon paste as working electrode, was connected to an electronic controller (LC 4, Bioanalytical systems).

Results

Kinetics of [³H]-prazosin binding

A time course study of specific [³H]-prazosin binding to myocardial membranes was performed at 25°C

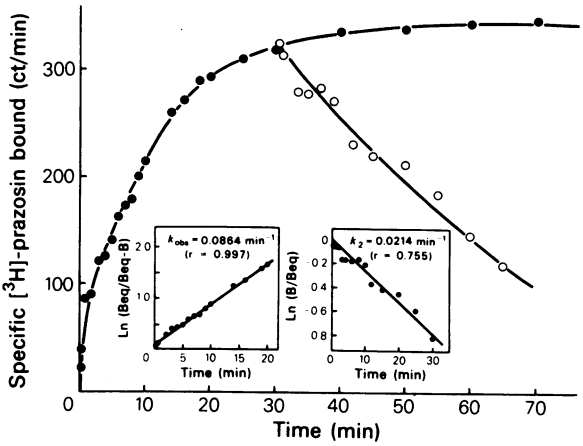

Figure 1 Time course of association (●) and dissociation (○) of [³H]-prazosin binding to cardiac membranes. Incubation was performed with [³H]-prazosin 0.59 nM at 25°C. Dissociation was induced by addition of 50 μM phentolamine after 30 min incubation. Inset: Determination of the pseudo first order rate constant for dissociation (k_{obs}) and of the first-order rate constant for dissociation (k_2). Beq represents specific binding at equilibrium and B specific binding at a given time. The slope was obtained by linear regression analysis.

using an initial concentration of the tritiated ligand of 5.9×10^{-10}M. Steady state was reached after approximately 30 min and was maintained up to 60 min indicating neither loss of binding sites nor degradation of the ligand during incubation. Excess phentolamine (50 μM final concentration) added after equilibrium was reached, progressively displaced the bound [³H]-prazosin. The second order rate constant for association (k_1) was determined as described by Williams *et al.* (1976) using the equation:

$$k_1 = \frac{k_{obs} - k_2}{[[^3H]\text{-prazosin}]} = 1.1 \times 10^{+8} M^{-1} min^{-1}$$

where k_{obs} is the pseudo-first order rate constant for association and k_2 is the rate constant for dissociation (Figure 1). The ratio $k_2/k_1 = 1.94\ 10^{-10}$M is a kinetically derived estimate of the equilibrium dissociation constant.

Number and affinity of binding sites

Specific binding of [³H]-prazosin to cardiac membranes at 25°C was a saturable process with a half-maximal saturation occuring at about 0.2 nM [³H]-prazosin (Figure 2). Non-specific binding increased linearly with the [³H]-prazosin concentration and represented 5 to 40% of total binding (0.1 to 5.0 nM). Scatchard analysis of the concentration-dependent binding curve indicates a single population of binding sites with a dissociation constant (k_D) of

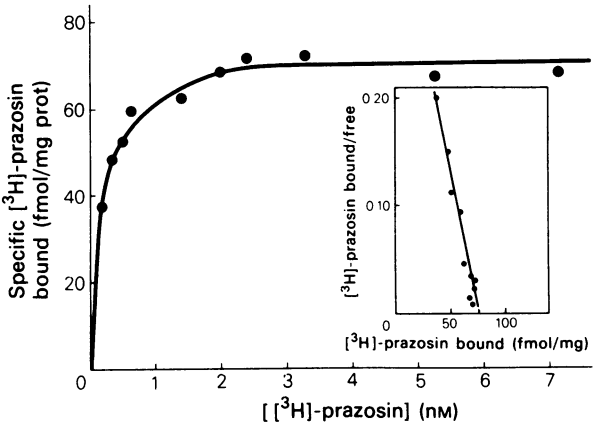

Figure 2 Specific binding of [^{3}H]-prazosin to rat cardiac membranes as a function of increasing concentrations. Membranes were incubated at 25°C for 40 min. Inset: Scatchard analysis of [^{3}H]-prazosin binding data.

$1.92 \pm 0.50 \times 10^{-10}$M and a maximal number of binding sites of $75.7 + 11.2$ fmol per mg of protein (mean ± s.e.mean). No evidence of cooperative interactions is apparent from Figure 2, in agreement with a Hill plot of [^{3}H]-prazosin binding which has a slope of 0.97. Specific binding was linear with increasing protein concentration up to 3 mg/ml.

Specificity of [^{3}H]-prazosin binding

The specificity of [^{3}H]-prazosin binding was studied using α-adrenoceptor agonists and antagonists (Table 1). The order of potency of phenylethylamines in inhibiting [^{3}H]-prazosin binding was: (−)-adrenaline > (−)-noradrenaline > phenylephrine >> isoprenaline in agreement with an α-adrenoceptor binding site (Ahlquist, 1948). The (+)-stereoisomers of adrenaline and noradrenaline are respectively 25 and 51 times less potent in inhibiting [^{3}H]-prazosin binding than the (−)-stereoisomers (Figure 3). The rank order of α-adrenoceptor agonists for [^{3}H]-prazosin binding sites was as follows: (−)-adrenaline > clonidine > (−)-noradrenaline

Table 1 Inhibition of [^{3}H]-prazosin binding by various pharmacological agents

Agent	K_1(nM)
α-Antagonists	
Prazosin	0.36
WB 4101	2.3
Dihydroergocryptine	5.4
Indoramin	9.6
Phentolamine	29
Yohimbine	560
Piperoxan	1,000
α-Agonists	
(−)-Adrenaline	1,360
Clonidine	1,390
(−)-Noradrenaline	3,200
Phenylephrine	7,400
S 3341	40,000
(±) α-Methylnoradrenaline	79,000
Other agents	
Dopamine	> 100,000
Isoprenaline	> 100,000
Propranolol	> 100,000

Cardiac membranes were incubated at 25°C for 40 min with 0.57 ± 0.77 nM [^{3}H]-prazosin and five or more concentrations of each drug. K_i values were calculated from the equation (Cheng & Prussof, 1973):

$$K_i = \frac{IC_{50}}{1 + \frac{C}{K_D}}$$

where IC_{50} represents the concentration of each agent which inhibits 50% of the specific binding, C, the [^{3}H]-prazosin concentration and K_D the dissociation constant of [^{3}H]-prazosin.
Values given are the means of three to six separate experiments, each performed in duplicate.

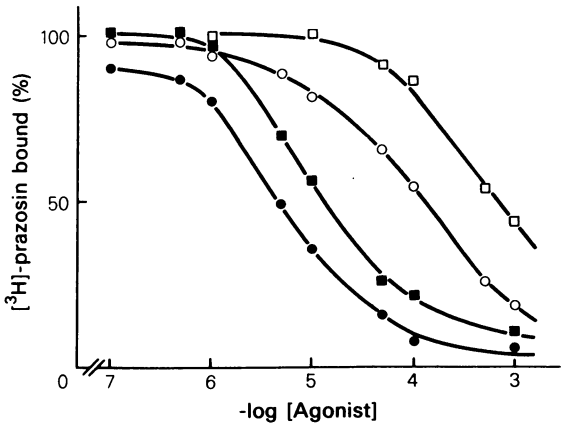

Figure 3 Inhibition of specific binding by (−)-adrenaline (●), (+)-adrenaline (○), (−)-noradrenaline (■) and (+)-noradrenaline (□) at increasing concentrations. Cardiac membranes were incubated at 25°C for 40 min with [^{3}H]-prazosin 0.65 nM in the absence and in the presence of competing agents. Results are expressed as the % of [^{3}H]-prazosin specifically bound in the absence of competitor.

> phenylephrine > S3341 > (±) α-methyl noradrenaline > dopamine. In contrast to agonists, the α-adrenoceptor antagonists had much higher affinities for [^{3}H]-prazosin binding sites. The rank order was prazosin > WB 4101 > dihydroergocryptine > indoramin > phentolamine > yohimbine > piperoxan.

Comparison of [^{3}H]-prazosin and [^{3}H]-dihydroergocryptine binding in rat heart

Comparative concentration-dependent binding studies were performed in parallel with [^{3}H]-prazosin and [^{3}H]-dihydroergocryptine using the same membrane preparation from hearts of non-treated 3 week old rats (Figure 4). The dihydroergocryptine binding curve exhibited the same break as previously observed (Figure 5), which has been attributed to the heterogeneity of sites (Guicheney *et al.*, 1978). The Scatchard representation of this curve shows a first linear component with a B_{max} of 100 fmol per mg of protein and a K_D = 2.2 nM. This high affinity component (represented on Figure 4 by open circles) corresponds to cardiac α_1-adrenoceptors. Indeed, in a previous study, we showed that the first component was competitively inhibited by a preferential α_1-antagonist such as ARC 239 (Guicheney *et al.*, 1978). In the present study, it was found that [^{3}H]-prazosin binds to only one class of α-adrenergic site with the specificity of α_1-adrenoceptors and furthermore that the number of [^{3}H]-prazosin binding sites (114.7 ± 4.9 fmol/mg prot.) was the same as for the high affinity binding component of [^{3}H]-dihydroergocryptine (98.0 ± 12.1 fmol/mg prot.).

Since presynaptic α_2-adrenoceptors have been demonstrated (Starke, 1972a,b; Yamaguchi, De Champlain & Nadeau, 1977) in the heart, we treated neonatal rats with 6-hydroxydopamine and assessed the binding of [^{3}H]-prazosin and [^{3}H]-dihydroergocryptine in this tissue. No variation in

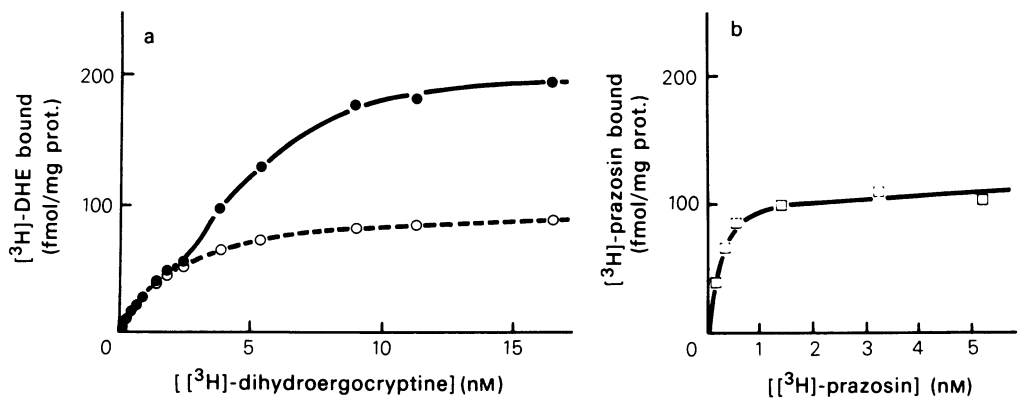

Figure 4 Specific binding of (a) [^{3}H]-dihydroergocryptine ([^{3}H]-DHE, ●) and (b) [^{3}H]-prazosin (□) to cardiac membranes as a function of increasing concentrations of the radioligands. Binding of [^{3}H]-dihydroergocryptine on α_1-adrenoceptors (○) was calculated from the formula:

$$B_{\alpha 1} = \frac{B_{\alpha 1 max}}{1 + \frac{K_{D\alpha 1}}{[[^3H]\text{-DHE}]}}$$

$B_{\alpha 1 max}$ and $K_{D\alpha 1}$ are determined from the first linear component of Scatchard representation of [^{3}H]-dihydroergocryptine concentration-dependent binding curve.

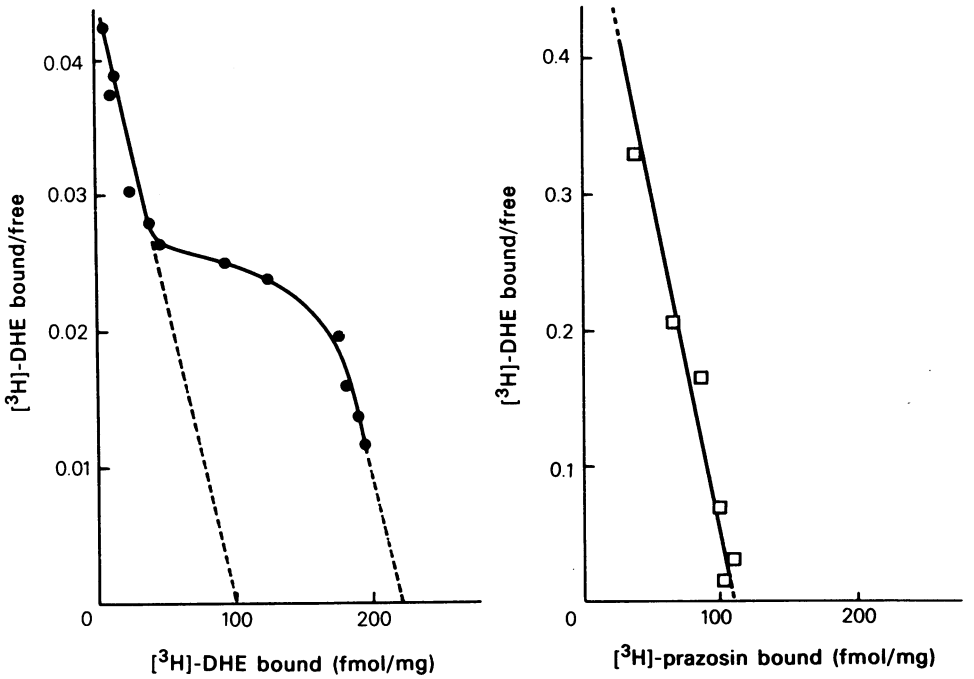

Figure 5 Scatchard analysis of the specific [^{3}H]-dihydroergocryptine [^{3}H]-DHE and [^{3}H]-prazosin binding; data presented in Figure 4.

binding characteristics was detected with either [^{3}H]-prazosin or [^{3}H]-dihydroergocryptine, although the cardiac noradrenaline content was markedly decreased from 1.98 ± 0.21 ng/mg tissue to 0.26 ± 0.03 ($P < 0.001$).

Discussion

Under the present experimental conditions, [^{3}H]-prazosin appears to bind specifically and with very high affinity according to the law of mass action to only one class of α-adrenoceptors in rat cardiac membranes. The nature of these α-adrenoceptors was determined by comparing the effect of α_1- and α_2-adrenoceptor agents on the binding of [^{3}H]-prazosin. Antagonists were more potent than agonists. Antagonist K_i values varied from 3.6×10^{-11}M to 1.0×10^{-6}M while agonist K_i values varied from 1.3×10^{-6}M to 7.9×10^{-5}M. The order of potency of the α-adrenoceptor agonists was: (−)-adrenaline > clonidine ≥ (−)-noradrenaline > phenylephrine > S-3341 > (±)α-methyl-noradrenaline. Phenylephrine is known to be a specific but weak α_1-agonist, less potent than noradrenaline in stimulating α_1-adrenoceptors in the heart and in the pulmonary artery (Starke, Endo & Taube, 1975; Verma & McNeill, 1976). (±)-α-Methyl noradrenaline, a preferential α_2-agonist is a poor competitor of [^{3}H]-prazosin binding, being less potent than the α_1-agonist, phenylephrine. On the other hand, clonidine was almost as potent as adrenaline which corresponds with its high potency at α-adrenoceptors. In addition to its well-known α_2 properties, clonidine stimulates the vascular α_1-adrenoceptors in pithed rats producing a vasoconstriction and a marked increase in blood pressure (Schmitt, 1977).

The α_1-nature of [^{3}H]-prazosin binding is further established by the relative potency of α-adrenoceptor antagonists as inhibitors of [^{3}H]-prazosin binding: prazosin > WB 4101 > dihydroergocryptine > indoramin > phentolamine >> yohimbine > piperoxan. The most potent are the most selective α_1-antagonists, prazosin and WB 4101. The weakest competitors were yohimbine and piperoxan, both preferential α_2-adrenoceptor antagonists.

The above data indicate that [^{3}H]-prazosin binds to sites possessing the characteristics of α_1-adrenoceptors. Twenty-one day old rats have a few more cardiac α_1-adrenoceptors than adult rats. The same phenomenon was observed for brain α- and β-adrenoceptors (Harden, Wolfe, Sporn, Perkins & Molinoff, 1977; Morris, Dausse, Devynck & Meyer, 1980).

[^{3}H]-dihydroergocryptine was previously shown to label two α-adrenoceptor binding sites (Guicheney *et al.*, 1978). The comparison between [^{3}H]-dihydroergocryptine and [^{3}H]-prazosin binding appears to confirm the α_1-nature of the first binding

component of [^{3}H]-dihydroergocryptine.

Indeed, the following observations further suggest that [^{3}H]-dihydroergocryptine at low concentrations and [^{3}H]-prazosin bind to the same receptors, i.e. to α_1-adrenoceptors: (i) the two radioligands bind according to the law of mass action with the same binding capacity, (ii) inhibition curves of [^{3}H]-prazosin binding by unlabelled prazosin and dihydroergocryptine are monophasic and the K_I values obtained are in agreement with the K_D values determined from binding studies for each radioligand used. Using preferential α_1 and α_2-antagonists, it has been shown that both α_1- and α_2-adrenoceptors are present in the uterus and that both are labelled by [^{3}H]-dihydroergocryptine (Hoffman, De Lean, Wood, Schocken & Lefkowitz, 1979) confirming the heterogeneity of [^{3}H]-dihydroergocryptine binding.

Various investigators have performed binding experiments in rats after 6-hydroxydopamine treatment, attempting to destroy presynaptic α_2-adrenoceptors. Story, Briley & Langer (1979) described a decrease in [^{3}H]-dihydroergocryptine binding sites in the heart ventricle of adult rats after 6-hydroxydopamine treatment. We were unable to produce similar results in neonatal rats. 6-Hydroxydopamine has been used in other tissues but the results of binding studies have been contradictory both in the periphery (U'Prichard & Snyder, 1979) and in the central nervous system (U'Prichard, Greenberg & Snyder, 1976; Skolnick, Stalvey, Daly, Hoyler & Davis, 1978; U'Prichard, Bechtel, Rouot & Snyder, 1979). Thus, 6-hydroxydopamine treatment may not be a satisfactory method for identifying presynaptic α_2-adrenoceptors.

In conclusion, [^{3}H]-prazosin appears to be a convenient ligand to label cardiac α_1-adrenoceptors. [^{3}H]-dihydroergocryptine binding suggests the presence of another class of α-adrenoceptor sites in rat heart which may be α_2-adrenoceptors but it is clear that the problem of characterization and localization of these receptors requires further study.

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