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ALPHA$_1$-ADRENERGIC RECEPTORS IN GUINEA PIG MYOCARDIUM: IDENTIFICATION BY BINDING OF A NEW RADIOLIGAND, (^{3}H)-PRAZOSIN

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SUMMARY: Binding of (^{3}H)-prazosin to adrenoceptors in guinea pig myocardial membranes was rapid, readily reversible, stereospecific and saturable. By Scatchard analysis (n = 6) Bmax was 58 fmol of (^{3}H)-prazosin bound/mg protein and the K_D was 0.58 nm. The Hill number was 1.05. Adrenergic agonists competed with (^{3}H)-prazosin as follows: (-)adrenaline > (-)noradrenaline > (-)phenylephrine >> (±)isoprenaline > (+)noradrenaline; antagonists competed in the order: non-radioactive prazosin > phentolamine >> piperoxan > yohimbine > sulpiride > propranolol. The K_D for beta-adrenoceptors assessed by (-^{3}H)-dihydroalprenolol was 0.86 nM and the Bmax (96 fmol/mg protein) was almost twice that of alpha-adrenoceptors. (^{3}H)-prazosin appears to be a useful radioligand for the study of post-synaptic (alpha$_1$) adrenoceptors in myocardial tissue.

INTRODUCTION

There is considerable evidence that the myocardium exhibits responses mediated by alpha-adrenoceptors, including alterations in contractility and changes in electrical properties, many of which are inhibited by specific alpha-adrenoceptor antagonists (1-3). Prior studies of alpha-adrenoceptors using radioligand binding methods usually have been performed in non-cardiac tissues but the existence of alpha-adrenergic receptors in rat myocardium has been demonstrated by Williams and Lefkowitz (1) using the radioligand (^{3}H)-dihydroergocryptine ((^{3}H)-DHE).

It has been shown that the alpha-adrenergic blocking agent prazosin is devoid of prejunctional (alpha$_2$) receptor affinity (4) and this compound has been characterised as a post-junctional (alpha$_1$) receptor blocking agent (5).

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Recently, high specific activity (^{3}H)-prazosin has been synthesised. Our purpose was to characterize for the first time alpha$_1$-adrenergic receptors in particulate fractions from guinea pig myocardium using this new radioligand and to compare the alpha$_1$- with the beta-adrenoceptor populations in the same preparation using a previously well characterised radioligand, (-^{3}H)-dihydroalprenolol ((-^{3}H)-DHA).

MATERIALS AND METHODS

(^{3}H)-prazosin was prepared at the Radiochemical Centre, Amersham, England by the reduction of bromoprazosin with tritium gas, yielding a specific activity of 33 Ci/nmol. Its radiochemical purity was greater than 98% by thin layer chromatography on silica gel and ethyl acetate : methanol : diethylamine (80 : 20 : 1) Rf 0.7 and ether : isopropylamine (95 : 5) Rf 0.2. Thin layer chromatography before and after incubation with tissue homogenates revealed no alteration in a homogenous peak, indicating no evidence of metabolism of the radioactive compound. (-^{3}H)-DHA was obtained from the same source and had a specific activity of 40 Ci/mmol. Its radiochemical purity was greater than 95% in 3 different solvent systems. Both compounds were stored at -80^{o}C and immediately prior to use were appropriately diluted in cold incubation buffer (50 mmol Tris-HCl, pH 7.5).

Male Dunkin-Hartley guinea pigs weighing 400-600 g were killed by cervical dislocation and the hearts rapidly removed, dissected free of pericardium, fat and great vessels and placed in 10 vol of ice cold 0.32 M sucrose. The tissue was homogenised at speed 7 for 15 sec using a Polytron homogeniser. Sufficient tissue was usually available from each heart for an individual experiment, but where necessary pooled tissue from 2 animals was used. Both atria and both ventricles were used for the preparation.

The homogenates were then centrifuged at 500 x g for 10 min at 4^{o}C. The pellets consisting of fibrous tissue, red cell and other high density debris were discarded and the supernatant recentrifuged twice at 50,000 x g for 15 min at 4^{o}C. The resulting pellets were washed in cold incubation buffer each time and were finally resuspended in cold buffer at an approximate concentration of 1 mg of protein/ml after filtration through fine nylon mesh. Protein was determined by the method of Lowry et al (6).

The membrane preparation was incubated for 15 min at 25^{o}C with either (^{3}H)-prazosin or (-^{3}H)-DHA. In preliminary experiments this duration of incubation was found to yield the optimum amount of specific binding for both radioligands. To each tube was added 0.8 ml of the membrane preparation, 0.1 ml of an appropriate concentration of radioligand and an additional 0.1 ml of either buffer or non-radioactive antagonist or agonist to make a total incubation mixture of 1.0 ml. Incubations with adrenergic agonists were carried out in the presence of 10^{-4}M pargyline and 0.1% ascorbic acid. Incubations were terminated by rapid vacuum filtration of the entire mixture through Watman GF/B glass fibre filters, which were immediately washed 3 times with 6 ml of ice cold incubation buffer.

Filters were placed in a liquid scintillation medium and radioactivity determined in a Packard liquid scintillation spectrometer at an efficiency of 40%. Specific binding of (^{3}H)-prazosin was taken as the amount of radioactivity bound to membranes that could not be displaced by either 10^{-5}M phentolamine or 10^{-6}M non-radioactive prazosin. Specific binding ranged from 60 to 80% of the total binding at final (^{3}H)-prazosin incubation concentrations of

0.1 to 0.5 nM. For (-^{3}H)-DHA specific binding was defined as that displaced by 10^{-5}M (dl)-propranolol and ranged from 60 to 80% of the total binding at final (-^{3}H)-DHA incubation concentrations of 0.1 to 0.4 nM. Counts bound to the filters in the absence of membranes ranged from 0.5 to 1.4% of the total counts after the filters were washed and counted in a manner identical to that used in the presence of membranes. For each data point in each experiment binding was carried out in triplicate.

RESULTS AND DISCUSSION

The binding of (^{3}H)-prazosin to cardiac membrane fractions was a saturable process of high affinity (Fig. 1A). Scatchard analysis revealed a linear plot suggesting a single class of binding sites. The calculated number of binding sites (Bmax) was 58 fmol of (^{3}H)-prazosin bound per mg protein at

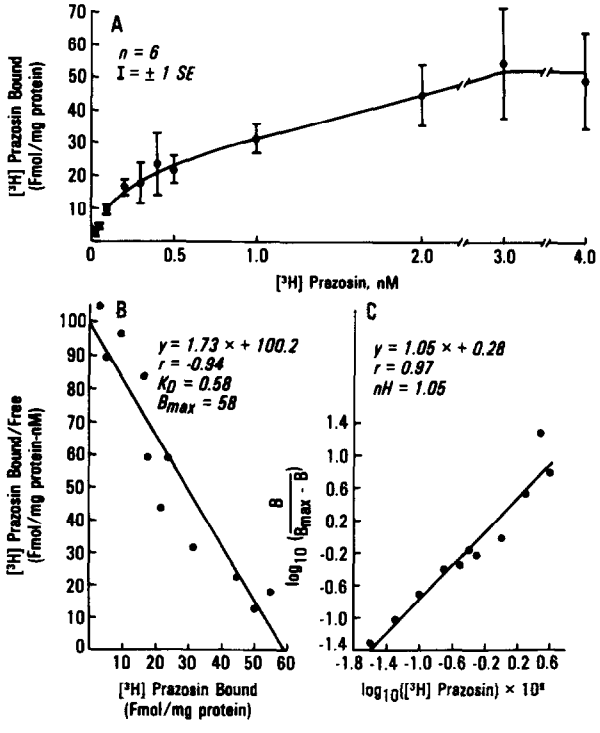

Figure 1

A. Specific (^{3}H)-prazosin binding with increasing concentrations of (^{3}H)-prazosin to guinea pig heart membranes.

B. Scatchard analysis of the data in A. The slope of the plot, $-1/K_D$ was determined by linear regression analysis and the number of binding sites, Bmax, computed from the intercept of the plot with the abscissa. For this analysis K_D = 0.58 nM and Bmax = 58 fmol/mg protein. When values for each experiment (n = 6) were computed individually and averaged, results were similar (K_D = 0.53 ± 0.17 nM, Bmax = 65 ± 26 fmol/mg protein). As indicated in the text, a Scatchard plot for (-^{3}H)-DHA binding gave a K_D of 0.86 nM and a Bmax of 96 fmol/mg protein. Corresponding values when data for each experiment (n = 6) were computed individually and averaged were similar (0.76 ± 0.17 nM and 100 ± 21 fmol/mg protein).

C. Hill plot of the data.

saturation and the K_D calculated from the negative reciprocal of the slope of the line was 0.58 nM (Fig. 1B). A Hill plot was linear with a slope of 1.05, suggesting the absence of cooperative interactions (Fig. 1C).

Kinetic analysis of the binding of (^{3}H)-prazosin showed it to be a rapid process with half maximal binding by 1 min at 25°C. Binding remained at steady state for at least 15 min and was rapidly reversed ($t_{\frac{1}{2}}$ = 1.2 min) by the addition of 10^{-4}M phentolamine (Fig. 2A). Calculations of pseudo first-order and second order rate constants are shown in Figs 2B and 2C.

Adrenergic agonists competed with (^{3}H)-prazosin for binding sites in the order expected for an alpha-adrenoceptor, i.e. (-)adrenaline > (-)noradrenaline

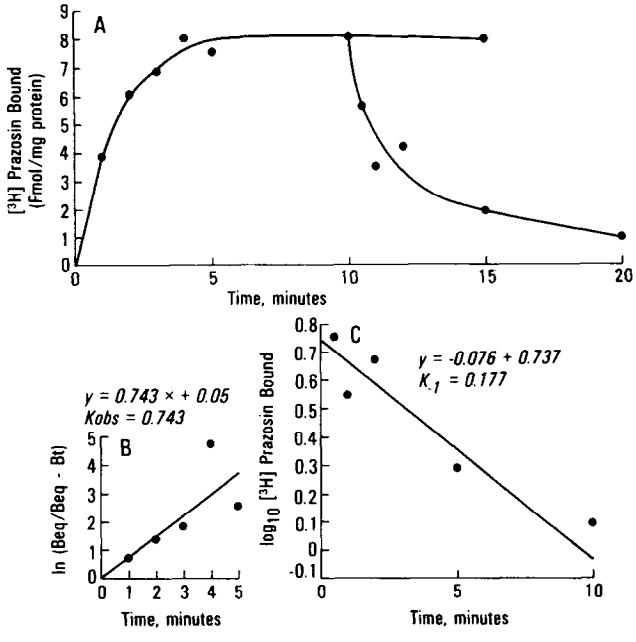

Figure 2

A. Kinetics of specific (^{3}H)-prazosin binding to guinea pig lung membranes at 25°C at a concentration of 0.26 nM (n = 4). Reversibility of binding was measured after the addition of 10^{-5}M phentolamine.

B. Pseudo-first order association plot with a slope determined by linear regression analysis, equal to the observed rate constant K_{ob}.

C. First order plot of dissociation with a slope of $k_{-1}/-2.303$ where k_{-1} is the first order rate constant for the reverse reaction. Calculation of the association constant (k_1) using the formula (7) $(k_{ob} - k_{-1})/(L)$ where (L) is the final incubation concentration of radioligand used in these experiments (0.26 nM), revealed a value of 2.17. The K_D by kinetic analysis was then determined from the ratio k_{-1}/k_1 and gave a value of 0.081 nM which is lower than that determined by saturation experiments and is consistent with results reported by others when these two methods of estimating K_D are compared (8,9).

TABLE 1

	IC_{50} (M)	K_i (nM)	Ratio of Potency
ANTAGONIST			
Prazosin	5.0×10^{-10}	0.33	1.0
WB 4101	1.8×10^{-9}	1.2	0.28
Indoramin	5.6×10^{-9}	3.7	0.089
Phentolamine	1.7×10^{-7}	126	0.0026
Mepyramine	1.7×10^{-6}	1264	0.00026
Methysergide	1×10^{-5}	7434	0.000044
Piperoxan	3×10^{-5}	22304	0.000015
Yohimbine	8×10^{-5}	59480	0.0000055
Sulpiride	2.5×10^{-4}	185873	0.0000018
Propranolol	2.7×10^{-4}	200743	0.0000016
AGONIST			
(-)Adrenaline	5×10^{-6}	3.7	1.0
(-)Noradrenaline	5×10^{-5}	37.2	0.10
Phenulephrine	8×10^{-5}	59.4	0.06
(±)Isoprenaline	9×10^{-4}	669	0.006
(+)Noradrenaline	1×10^{-3}	743	0.005

K_i values were determined from the equation $K_i = IC_{50}/(1 + (L)/K_D)$, where IC_{50} is the molar concentration of the agent causing 50% inhibition of specific binding as determined by log probit analysis. (L) is the concentration of (^{3}H)-prazosin used in the assay (0.26 nm) and the K_D was determined by Scatchard analysis (0.58 nM). Each value is the mean of 3-5 experiments, each performed in triplicate.

>> (-)phenylephrine > (±)isoprenaline > (+)noradrenaline (Table 1). Stereospecificity was demonstrated by the considerably greater potency of (-)noradrenaline compared with (+)noradrenaline with Ki values of 37.2 and 743 μM,

respectively and relative potency ratios compared with (-)adrenaline of 0.10 and .005, respectively (Table 1).

Adrenergic antagonists also competed for (^{3}H)-prazosin binding sites with expected specificity, phentolamine exhibiting a markedly greater affinity for these binding sites than propranolol (Table 1). Non-radioactive prazosin was the most potent inhibitor of (^{3}H)-prazosin binding while an antihistamine, mepyramine, and an antiserotonin agent, methysergide, both exhibited considerably weaker activity. Both piperoxan and yohimbine, which have a higher affinity for presynaptic than for post-synaptic sites, showed weak binding affinity as did sulpiridine, a specific dopamine antagonist.

For comparison the number and affinity of binding sites of (-^{3}H)-DHA to cardiac membranes was measured employing the identical preparation and incubation conditions used to characterize (^{3}H)-prazosin binding. As demonstrated by others (1) and confirmed by us, binding of (-^{3}H)-DHA was saturable, readily reversible and stereospecific, l-propranolol being 50x more potent than d-propranolol. By Scatchard analysis the number of (-^{3}H)-DHA binding sites exceeded that observed for (^{3}H)-prazosin (Bmax = 96 fmol/mg protein), giving a ratio of beta- to alpha-adrenergic binding sites of 1.6 : 1. The K_D for (-^{3}H)-DHA by Scatchard analysis was 0.86 nM.

To test the relation between alpha- and beta-adrenoceptors in another species, membranes prepared in an identical manner from normal golden hamster hearts were also examined (n = 4). Scatchard analysis confirmed the high affinity binding characteristics of (^{3}H)-prazosin (K_D = 0.07 nM), while the K_D value for (-^{3}H)-DHA was similar to that of the guinea pig (1.23 nM). The Bmax value for (^{3}H)-prazosin was low (3.8 fmol/mg protein),suggesting that in this species as well there are an excess number of beta- compared with alpha-adrenoceptors.

Previous studies using an alpha-adrenergic antagonist to label alpha-adrenoceptors have employed (^{3}H)-DHE exclusively as the radioligand (1,7,8,10,11)

(^{3}H)-DHE may label serotonin and dopamine receptors in brain (8) and labels both pre- and post-synaptic alpha-adrenergic receptors in cardiac and other tissues (10,11). In the only other study employing (^{3}H)-prazosin, Greengrass and Bremner also reported a K_D that was subnanomolar (0.29 nM) in a rat brain tissue preparation and provided additional evidence that (^{3}H)-prazosin binds to post-synaptic ($alpha_1$) receptors (12). They found that piperoxan and yohimbine were considerably less potent than phenoxybenzamine and indoramin in inhibiting (^{3}H)-noradrenaline binding. Our own observations in guinea pig heart are consistent with these results and indicate that both piperoxan and yohimbine, both of which have a greater affinity for presynaptic ($alpha_2$) receptors, are 177 to 472-fold less potent, respectively in competing for (^{3}H)-prazosin binding sites than is phentolamine, which binds to both $alpha_1$ and $alpha_2$ subclasses of alpha adrenoceptor (Table 1).

For comparison, (^{3}H)-DHA was used to bind to sites in the same membrane preparations having the characteristics of beta-adrenergic receptors. The K_D of 0.86 nM is in agreement with the value reported by U'Prichard et al (9) in a rat heart preparation (0.61 nM). We observed a relative excess of beta- compared with alpha-adrenoceptor binding sites in both guinea pig and hamster myocardial membranes and have noted a similar relation in membranes prepared from guinea pig and human lungs. The relatively smaller number of alpha- in relation to beta-adrenergic receptors identified by (^{3}H)-prazosin binding as compared with a ratio of one when (^{3}H)-DHE was used as the radioligand (1) could be a consequence of binding predominantly to postsynaptic ($alpha_1$) receptors with relatively little binding to presynaptic ($alpha_2$) receptors. Thus, Briley et al have shown that destruction of noradrenergic nerve terminals by 6-hydroxydopamine in the rat reduced (^{3}H)-DHE binding by 43%, suggesting that approximately half the binding with this ligand is to postsynaptic receptors (10). In addition to the apparent difference in binding preference and characteristics of these two radioligands, other possible explanations for this discrepancy and for the low K_D noted for (^{3}H)-prazosin as compared with

(^{3}H)-DHE (1) include species difference, or differences in the methods of membrane preparation and assay.

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