Vol. 91, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1979 Pages 200-206

[^{3}H]Dihydroalprenolol Binding Sites in Rat Myocardium: Relationship Between a Single Binding Site Population and the Concentration of Radioligand

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Received September 15, 1979

Summary: [^{3}H]Dihyroalprenolol bound to a single population of high affinity sites in rat myocardial membranes when the concentration of the radioligand was below 5 nM. These sites displayed characteristics which would be expected of binding to the β-receptor. Kinetic- and Scatchard-derived dissociation constants were 0.6 and 2.0 nM, respectively. Binding was to a limited number of sites, 60 fmols/mg protein. Scatchard analysis using radioligand concentrations in excess of 5 nM resulted in concave upward plots suggestive of more than one population of binding sites. The lower affinity sites (labeled at high radioligand concentration) were non-stereospecific in nature and became a progressively larger fraction of "specific binding" as the concentration of dihydroalprenolol was increased above 5 nM.

Introduction: Many of the physiological responses of the heart to adrenergic agents appear to be mediated through the β-adrenergic receptor and the subsequent cyclic AMP cascade (1,2). A number of studies to date have attempted to label the cardiac β-receptor with [^{3}H]dihydroalprenolol (3-11). These studies have generally reported equilibrium dissociation constants (K_ds) for the interaction of the cardiac receptor with dihydroalprenolol of 10 - 15 nM (3-8), although U'Prichard et al., (9) and more recently Williams et al., (10, 11) have reported K_ds of 1 - 2 nM for the cardiac receptor. Binding studies in the heart have been hindered by the low density of β-receptors achieved in most membrane preparations which has necessitated the use of high radioligand concentrations to measure binding and by large amounts of "non-specific" binding observed with the currently available radioligands.

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0006-291X/79/210200-07$01.00/0

The present report gives both kinetic and equilibrium data to support the higher affinity (K_d = 2 nM) site reported in the literature (9-11) as the β-receptor and the lower affinity (K_d = 10 -15 nM) site (3-8) as an artifact of linear regression analysis of at least 2 different sites with markedly different properties.

Materials and Methods: Male Holtzman rats from Holtzman inc., Madison, Wisc. weighing 200-250 g were anesthetized with ether and the hearts were rapidly excised and perfused through the aortic stalk with room temperature saline (0.9%) to remove blood elements. Perfused hearts were then trimmed of both atria, fat and great vessels and the left chamber was opened through the left free wall of the ventricle. The closely adherent epicardium could than be removed by rolling the opened ventricle between the folded sheets of a Whatman filter disc. Ventricles with epicardia removed were then placed in cold (4°C) homogenizing buffer consisting of 0.25 M sucrose, 5 mM TRIS-HCl, 1 mM $MgCl_2$, pH 7.4 at 4°C. All subsequent preparatory steps were performed at 4°C. Ventricles were finely minced and homogenized in 10 volumes of homogenizing buffer with a Polytron PT-10 for 15 sec each at low, moderate, and high speeds with 30 sec allowed between pulses for cooling.

Homogenates were initially spun for 10 min at 460 x g. The first pellet, consisting predominantly of cell debris, nuclei and contractile proteins was discarded and the resulting supernatant passed through 4 layers of cheese cloth and recentrifuged for 10 min at 30,000 x g. This pellet was then twice resuspended in 10-15 volumes (original wet weight) of incubation buffer consisting of 50 mM TRIS-HCl, 5 mM $MgCl_2$, pH 7.4 at 4°C and centrifuged for 10 min at 30,000 x g. The final washed pellet was resuspended in 1 ml of incubation buffer containing 1 mM ascorbic acid and 0.1 mM pyrocatechol for every g wet weight of starting material.

(-)[^{3}H]Dihydroalprenolol binding assays were performed in triplicate at 22°C by adding 50 µl of the membrane suspension (160-240 µg protein) to 12 x 75 mm polypropylene tubes containing [^{3}H]dihydroalprenolol (specific activity 51.1 Ci/mMol, obtained from New England Nuclear) and were for 15 minutes with shaking in a final volume of 250 µl of 50 mM TRIS-HCl, 5 mM Mg Cl_2, 1 mM ascorbic acid, and 0.1 mM pyrocatechol, pH 7.4 at 22°C. Reactions were terminated by placing a 200-µl aliquot of the reaction mixture into 3 ml of cold incubation buffer and immediately filtering the suspension through a Whatman GF/C glass fiber filter. Filters were then washed with an additional 9 ml of cold incubation buffer, placed into scintillation vials, oven dried, and counted in 10 ml of Triton-toluene based scintillation fluid. Counting efficiencies obtained were generally 46-50%. Non-specific binding was defined as the amount of radioligand bound in the presence of 10^{-5}M (±)propranolol. Specific binding, which represented the difference between total and non-specific binding was 65-80% of total binding at radioligand concentrations below 5 nM.

Results and Discussion: Experiments reported here were done at 22°C, although qualitatively similar results can be obtained at 37°C. [^{3}H]Dihydroalprenolol binding to rat myocardial preparations was rapid, reaching equilibrium within 5-6 minutes (fig 1A). The pseudo-first order rate constant, k_{ob}, for the

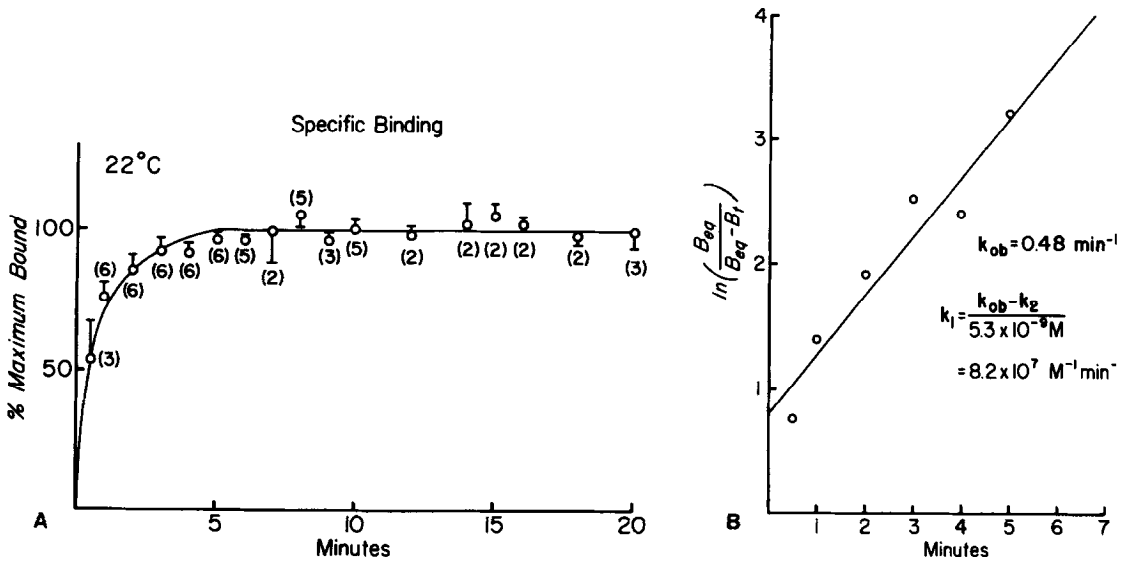

Fig. 1, A. Kinetics of specific [^{3}H]dihydroalprenolol binding to myocardial membranes. At designated times 200-µl aliquots were removed from a starting incubation volume of 3.0 ml. Numbers in parenthesis represent the number of different experiments in which aliquots were assayed at that particular time.

B. Pseudo-first order rate plot of the binding data presented in fig. 1,A, taken prior to equilibrium.

association reaction can be obtained from the slope in figure 1B, taken from specific binding data prior to equilibrium. The second order rate constant, k_1, can then be calculated by $k_1 = (k_{ob}-k_2)/(DHA)$, where (DHA) is equal to the concentration of [^{3}H]dihydroalprenolol in the reaction, and k_2 is the dissociation rate constant which was determined independently (fig 2). The k_1 for the reaction of [^{3}H]dihydroalprenolol with the myocardial receptor was 8.2 x 10^7 $M^{-1}min^{-1}$.

Dissociation of specifically bound [^{3}H]dihydroalprenolol from myocardial membranes was measured by "infinitely diluting" (100-fold excess buffer) an equilibrated mixture of [^{3}H]dihyroalprenolol and myocardial membranes. Dissociation at 22°C was rapid and first order, characterized by a rate constant, k_2, of 0.050 min^{-1} (fig. 2 inset) which agreed well with 4 other determinations, 0.048 ± 0.004 min^{-1} (mean ± standard error).

The ratio of the rate constants, k_2/k_1, provides an estimate of the equilibrium dissociation constant, K_d, for the interaction between [^{3}H]dihydroal-

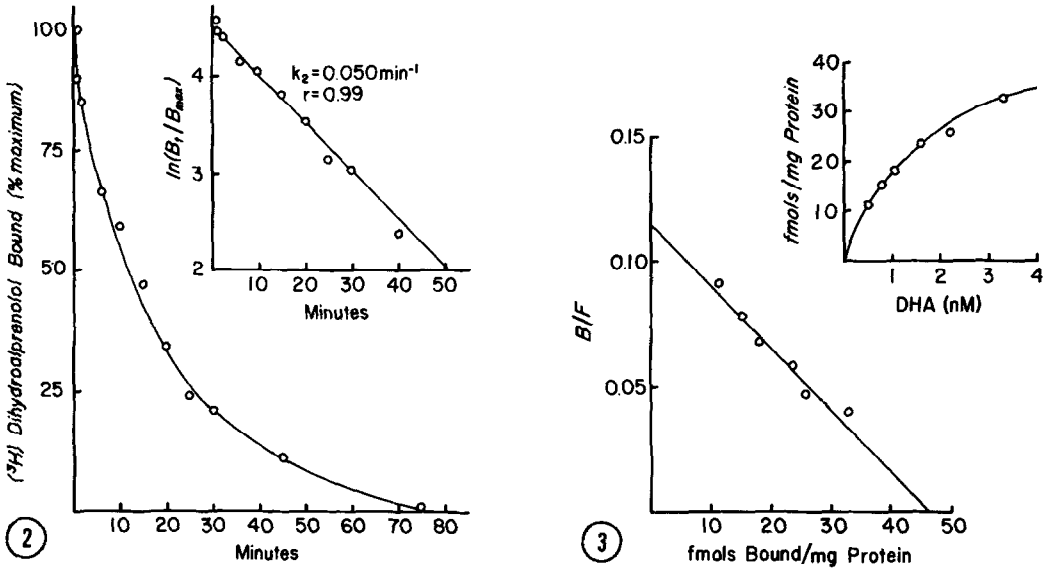

Fig. 2. Dissociation of specifically bound [^{3}H]dihydroalprenolol from myocardial membranes. Aliquots (1.2 ml) of equilibrated radioligand-receptor preparations incubated in the presence and absence of 10^{-5}M (±)propranolol were diluted 100-fold ("infinite dilution") with buffer and 10-ml aliquots were withdrawn at the indicated times and assayed for total and non-specific binding. Inset: Same data plotted as a first-order rate plot. The slope is equal to the dissociation rate constant (k_2).

Fig. 3. Scatchard plot of specific [^{3}H]dihydroalprenolol binding to myocardial membranes. Membranes have been incubated with [^{3}H]dihydroalprenolol at concentrations between 0.5 -5.0 nM. The correlation coefficient for the line drawn by linear regression in this experiment was 0.98.

prenolol and the cardiac binding site. From values of 0.048 min^{-1} for the k_2 and 8.2 x 10^7 $M^{-1}min^{-1}$ for the k_1, the kinetically determined K_d was calculated to equal 0.6 nM.

[^{3}H]Dihydroalprenolol binding sites in the rat myocardial preparations appeared to be saturable at low concentrations (<5 nM) of radioligand as evidenced by the hyperbolic shape of the concentration-response curve (fig. 3 inset). However, at higher radioligand concentrations, specific binding continued to increase in an almost linear fashion. Scatchard analysis (12) using [^{3}H]dihydroalprenolol concentrations greater than 5 nM resulted in visibly curvilinear plots suggestive of either multiple binding sites or receptor site-to-site interactions (cooperativity) (13). Dissociation constants

calculated by linear regression of these curvilinear plots approached 10 nM, a value frequently cited in reports using cardiac tissue (3-8), and gave reasonably acceptable coefficients of linearity (0.8-0.9).

Considering the kinetically derived dissociation constant (k_2/k_1) of 0.6 nM and the hyperbolic concentration-response curve obtained at lower concentrations of [^{3}H]dihydroalprenolol, we have generally performed Scatchard analysis over a [^{3}H]dihydroalprenolol concentration range of 0.5 - 5.0 nM. Scatchard plots constructed in this manner are visibly linear (fig. 3) and of identical slope whether 10^{-5}M (±)propranolol or 10^{-3}M l-isoproterenol is used to measure non-specific binding. Equilibrium dissociation constants of 2.1 ± 0.3 nM and 2.0 ± 0.4 nM (mean ± standard error) for propranolol- and isoproterenol-displaceable binding, respectively, were obtained by Scatchard analysis (12) which is in reasonable agreement with the kinetically determined K_d and also with the lower K_ds reported by U'Prichard et al., (9) and Williams et al., (10,11). Total myocardial receptors measured from Scatchard plots constructed in this manner were 60 ± 6 fmols/ mg protein.

The characteristics of the myocardial sites labeled at the lower radioligand concentration were consistent with the β-receptor. Binding was strictly stereospecific, (-) isomers being on the order of 100-times more potent than the corresponding (+) isomers. Agonists competed for the [^{3}H]dihydroalprenolol binding sites in the potency order of isoproterenolol > epinephrine > norepinephrine. β-antagonists competed for the myocardial sites at much lower concentrations than did β-agonists, consistent with the potencies of these compounds in more physiological preparations. (data not shown).

Assuming the K_d of the high affinity [^{3}H]dihydroalprenolol binding site in rat myocardium to be 2 nM, Scatchard plots constructed over the concentration range of 0.5 -5.0 nM would span 0.25 -2.5 times the K_d of the receptor for the radioligand. Theoretically, at 5 nM [^{3}H]dihydroalprenolol, 71% of the available receptors would be occupied by the radioligand. Thus we feel that concentrations of dihydroalprenolol up to 5 nM, which are on the linear or

"high affinity" part of the Scatchard plot, are sufficient to extrapolate to receptor saturation in myocardial membranes.

Non-linearity in Scatchard plots obtained with [^{3}H]dihydroalprenolol concentrations in excess of 5 nM appear to be due to a second, lower affinity binding site. (10, 14). At higher radioligand concentrations and also at lower temperature (4°C) we have been able to label a second, lower affinity (K_d = 25-40 nM), non-stereospecific "acceptor site" in rat myocardial tissue that is present in at least 10-fold greater amounts than the higher affinity site described at low concentrations of radioligand (manuscript in preparation). This lower affinity site becomes a progressively larger fraction of "specific binding" as the concentration of [^{3}H]dihydroalprenolol is increased. Scatchard plots constructed at radioligand concentrations where this site becomes a significant fraction of "specific binding" will therefore be curvilinear and linear regression analysis of the data will have the effect of artifactually decreasing the apparent affinity and increasing the number of binding sites. A similar second class of [^{3}H]dihydroalprenolol binding sites of lower affinity lacking stereospecificity have recently been reported in the non-fusing BC_3H_1 muscle cell line by Mauger and Worcel (14).

Acknowledgement

We wish to thank Dr. Manuel Worcel for a copy of his manuscript prior to publication. This work was supported by a grant from N.I.H., grant number HL 19027 and a grant-in-aid by the Iowa Heart Association.

References

1. Tsien, R.W., (1977) Adv. in Cyclic Nucleotide Res. 8: 363-421.
2. Drummond, G.I., Severson, D.L., (1979) Circ. Res. 44: 145-153.
3. Alexander, R.W., Williams, L.T., Lefkowitz, R.J., (1975) Proc. Natl. Acad. Sci. USA 72:1564-1568.
4. Krawietz, W., Poppert, D., Erdmann, E., Glossmann, H., Struck, C.J., Konrad, C., (1976) Naunyn-Schmiedeberg's Arch. Pharmacol. 295: 215-224.
5. Williams, L.T., Lefkowitz, R.J., Watanabe, A.M., Hathaway, D.R., Besch, H.R., (1977) J. Biol. Chem. 252: 2787-2789.
6. Glaubiger, G., Lefkowitz, R.J., (1977) Biochem. Biophys. Res. Commun. 78: 720-725.

7. Glaubiger, G., Tsai, B.-S., Lefkowitz, R.J., Weiss, B., Johnson, E.M., (1978) Nature 273: 240-242.
8. Limas, C., Limas, C.J., (1978) Biochem. Biophys. Res. Commun. 83: 710-714.
9. U'Prichard, D.C., Bylund, D.B., Snyder, S.H., (1978) J. Biol. Chem. 253: 5090-5102.
10. Williams, R.S., Lefkowitz, R.J. (1978) Circ. Res. 43: 721-727.
11. Williams, R.S., Lefkowitz, R.J., (1979) Clin. Res. 27:216A.
12. Scatchard, G., (1949) Ann. N.Y. Acad. Sci. 51: 660-676.
13. Cuatrecasas, P., Hollenberg, M., (1976) Adv. Protein Chem. 30: 251-451.
14. Mauger, J-P., Worcel, M. (1979) Brit. J. Pharmacol. (in press).