# STEREOSPECIFIC [$^3$H](-)-ALPRENOLOL BINDING SITES, β-ADRENERGIC RECEPTORS AND ADENYLATE CYCLASE

Robert J. Lefkowitz, Chhabirani Mukherjee, Michael Coverstone and Marc G. Caron

Division of Cardiology, Department of Medicine and Department of Biochemistry
Duke University Medical Center, Durham, North Carolina 27710

Received July 29,1974

SUMMARY. The binding of [$^3$H](-)-alprenolol (a potent β-adrenergic antagonist) to sites in frog erythrocyte membranes has been studied by a centrifugal assay. The specificity of the binding sites is strikingly similar to what might be expected of the β-adrenergic receptor binding sites which mediate stimulation of adenylate cyclase by catecholamines in these membranes. The sites bind β-adrenergic antagonists and agonists with affinities which are directly related to their antagonist or agonist potency on the frog erythrocyte membrane adenylate cyclase. Binding shows strict stereospecificity with (-)-isomers exhibiting two orders of magnitude higher affinities than (+)-isomers. Dissociation constants for potent β-adrenergic antagonists are in the range of $10^{-9}$ - $10^{-8}$M whereas those for β-adrenergic agonists are about two orders of magnitude higher ($\geq 10^{-6}$M).

Dramatic advances in the identification and isolation of hormone and drug receptors have occurred in the past few years. Progress has been most rapid in the case of certain polypeptide hormones (1,2) and the nicotinic cholinergic receptor (3). These developments have been made possible by the availability of highly specific radioactive marker compounds which can be used for direct binding studies e.g. $^{125}$I-insulin (4), $^{125}$I-bungarotoxin (5), [$^3$H]vasopressin (6), etc. Such radioactive materials have facilitated the crucial first step in the process of hormone receptor isolation, that of receptor identification.

Progress in the field of adrenergic receptor identification and isolation has proceeded more slowly. Use of [$^3$H] labelled agonists such as epinephrine (7,8), norepinephrine (9-12) and isoproterenol (13-14) has permitted the study of sites possessing certain characteristics of β-adrenergic receptors (15,16). However, these sites have not exhibited a number of characteristics which might be expected of β-adrenergic receptors (15,16). In particular, whereas catecholamine action invariably is stereospecific, (i.e. the (-)-isomers are considerably more active than the (+)-isomers), [$^3$H]catecholamine binding is not. Secondly, affinity of [$^3$H]catecholamine binding sites for adrenergic antagonists is relatively low ($K_D \sim 10^{-4}$M).

Accordingly, we have been searching for a specific radioactive adrenergic ligand which would reflect in its binding characteristics stereospecificity and high affinity for the β-adrenergic receptors. In this communication

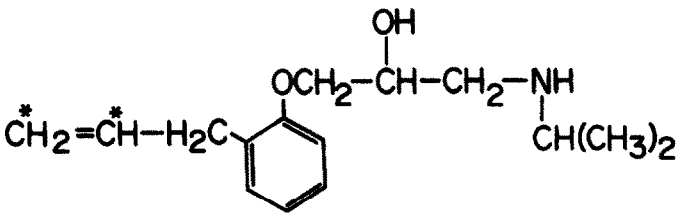

Fig. 1: Structural formula of [$^3$H](-)-alprenolol. "*" indicates the sites of tritiation.

---

we describe the characteristics of binding of [$^3$H](-)-alprenolol (a potent and specific β-adrenergic antagonist) to frog erythrocyte membranes.

MATERIALS AND METHODS. (-)- and (+)-alprenolol hydrochloride were gifts of Dr. Ablad, Hassle Pharmaceuticals, and (-)- and (+)-propranolol of Ayerst Pharmaceuticals. (-)-Isoproterenol bitartrate, (-)-norepinephrine bitartrate, phosphoenol pyruvate, pyruvate kinase, ATP and cAMP were from Sigma. Phentolamine was from Ciba. [$^3$H]cAMP (1-5 Ci/mmole) and [α-$^{32}$P]ATP (1-10 Ci/mmole) were from New England Nuclear Co. Alumina, neutral grade was from Nutritional Biochemicals. Grass frogs were from Carolina Biological or Nasco and Steinhilber.

(-)-Alprenolol hydrochloride (Fig. 1) (17) was tritiated to high specific activity (10 Ci/mmole) at New England Nuclear Co. by catalytic reduction of the unsaturated bond with tritium using paladium as the catalyst. Prior to each experiment, the labelled compound was purified twice by thin layer chromatography on silica gel plates (Baker-1BF) which were developed with acetone: benzene: acetic acid, 70:25:5.

Preparation of frog erythrocyte membranes - Blood from grass frogs maintained at room temperature was collected and the red cells washed three times with a solution of 110 mM NaCl, 10 mM Tris-HCl, pH 7.4. Cells were lysed in 5 mM Tris-HCl, pH 8.1 buffer and the membranes centrifuged at 18,000 xg for 15 minutes. The lysis process was repeated three times. Membranes were finally suspended in 75 mM Tris-HCl, 25 mM $MgCl_2$, pH 8.1 by homogenization.

Protein was measured by the method of Lowry, et al (18).

RESULTS. [$^3$H](-)-alprenolol bound to sites in the erythrocyte membranes. Equilibrium binding was attained within 10-15 minutes at 23° or 37° (data not shown). Several adrenergic antagonists were tested for their ability to compete for these binding sites (Fig. 2). Most potent were (-)-alprenolol and (-)-propranolol which displaced over the concentration range from $10^{-10}$M to $10^{-7}$M. By contrast the (+)-isomers of these antagonists had only about 1/100th the potency of the (-)-stereoisomers. The α-adrenergic antagonist phentolamine was without effect.

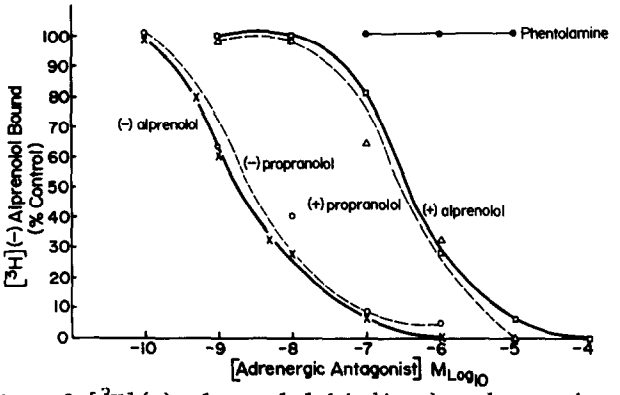


Fig. 2: Inhibition of [$^3$H](-)-alprenolol binding by adrenergic antagonists. 100-200 µl aliquots of membranes were incubated with [$^3$H](-)-alprenolol, $10^{-9}$ - $10^{-8}$M, in the presence or absence of unlabelled adrenergic agonists or antagonists, generally for 30 minutes at 23°C. At the completion of incubations duplicate 50 or 100 µl aliquots were layered over 300 µl of 10% sucrose in a Brinkman Microfuge. Bound [$^3$H](-)-alprenolol was sedimented to the bottom of the tubes whereas the free labelled material remained above the sucrose. After aspiration of the supernatant, pellets were solubilized overnight with 10% sodium dodecyl sulfate. The tips of the plastic microfuge tubes were then cut off into vials containing a triton-toluene scintillation fluid and counted in a Packard Liquid Scintillation Counter. In all experiments, "non-specific" binding was defined as that amount of [$^3$H](-)-alprenolol which was recovered in membrane pellets when incubations were performed in the presence of $10^{-6}$M (-)-propranolol or (-)-alprenolol. This "non-specific" binding (generally 1/4th - 1/6th of "specific binding") appeared to largely represent labelled material trapped in the membrane pellet during centrifugation. Each value is the mean of 3 - 4 separate experiments determined in duplicate or quadruplicate. "Control" binding was 0.2 pmoles [$^3$H](-)-alprenolol bound/mg membrane protein.

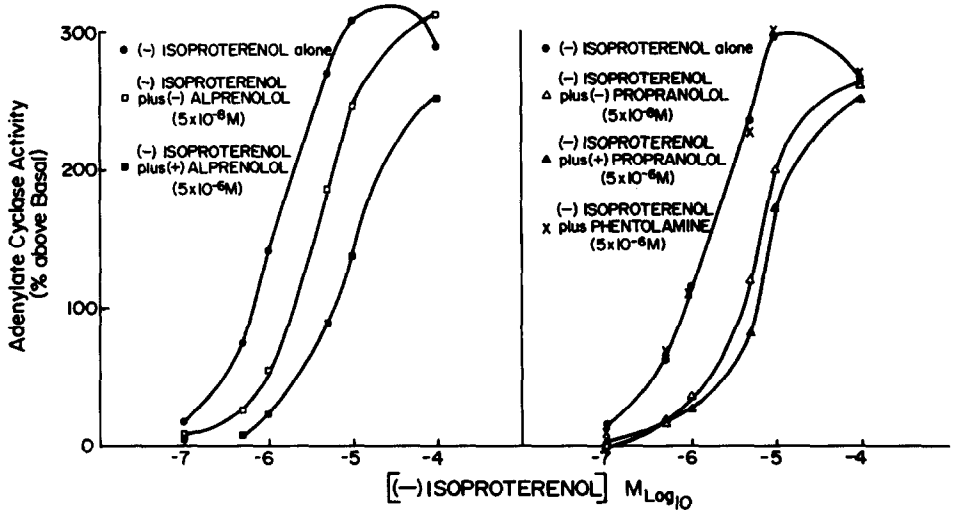


Fig. 3: Inhibition of isoproterenol stimulated frog erythrocyte membrane adenylate cyclase by adrenergic antagonists. Adenylate cyclase assays were performed as previously described (19,20) by following conversion of [$\alpha$-$^{32}$P]ATP (1.5 mM) to [$^{32}$P]cAMP in the presence of an ATP regenerating system. The labelled product was isolated on columns of neutral alumina (21) and product recovery on each column was monitored with [$^3$H]cAMP. Values shown are means of 3 experiments performed in duplicate. Basal adenylate cyclase was 272 pmoles cAMP generated/15 min/mg protein.

In oder to correlate the ability of the antagonists to block [$^3$H](-)-alprenolol binding with a measurable biological effect, each compound was also tested for its efficacy as an antagonist of isoproterenol stimulated adenylate cyclase in the membranes. Fig. 3 presents dose response curves for stimulation of the erythrocyte membrane adenylate cyclase by isoproterenol in the absence or presence of various concentrations of antagonists. There is a direct correlation of ability to competitively antagonize isoproterenol stimulation and [$^3$H](-)-alprenolol binding. Furthermore, data such as those presented in Fig. 3 permit the calculation of dissociation constants of each antagonist for the adenylate cyclase-coupled β-adrenergic receptors. The equation is, $K_D = \frac{[\text{antagonist}]}{\text{dose ratio}-1}$ where dose ratio refers to the ratio of equiactive concentrations of an agonist (isoproterenol) in the presence and absence of a given antagonist concentration (22). Such $K_D$'s are tabulated in Table 1. Close agreement was found between these values, and the concentrations of the antagonists which 50% displaced [$^3$H](-)-alprenolol from the binding sites (Table 1).

Fig. 4 A indicates that the β-adrenergic agonist (-)-isoproterenol also competed for the binding sites. Half maximal displacement occurred at $10^{-6}$M, precisely the concentration which half maximally activated the erythrocyte adenylate cyclase (Fig. 4 B). By contrast (-)-norepinephrine was much weaker in competing for the [$^3$H](-)-alprenolol binding sites and in activating the cyclase (Fig. 4 A and B).

DISCUSSION. Frog erythrocyte membranes were chosen for these initial studies because they contain a well characterized (20, 23-25) and highly catecholamine-responsive adenylate cyclase coupled to a typical β-adrenergic receptor. [$^3$H](-)-alprenolol was utilized for several reasons. Its affinity for the β-adrenergic receptors appears to be extremely high (Fig. 3), being equal to that of (-)-propranolol (17). The compound was available in stereoisomeric

| ANTAGONIST | $K_D$ FOR INHIBITION OF ISOPROTERENOL STIMULATED ADENYLATE CYCLASE | 1/2 MAXIMAL INHIBITION OF [$^3$H](-)-ALPRENOLOL BINDING |
|---|---|---|
| (-)-alprenolol | $1 \times 10^{-8}$M | $2 \times 10^{-9}$M |
| (-)-propranolol | $1 \times 10^{-8}$M | $3 \times 10^{-9}$M |
| (+)-alprenolol | $5 \times 10^{-7}$M | $3 \times 10^{-7}$M |
| (+)-propranolol | $7 \times 10^{-7}$M | $3 \times 10^{-7}$M |
| phentolamine | ----- | ----- |

Table 1: Inhibition of [$^3$H](-)-alprenolol binding and (-)-isoproterenol activated adenylate cyclase in frog erythrocyte membranes by β-adrenergic antagonists. ----- indicates no effect at concentrations up to $10^{-5}$M.

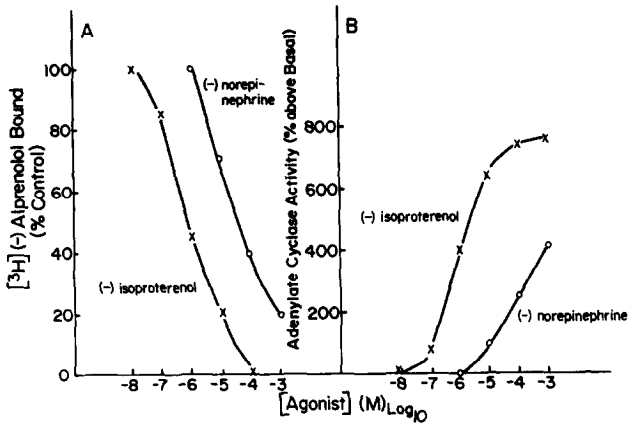


Fig. 4 (A) Inhibition of [$^3$H](-)-alprenolol binding by (-)-isoproterenol and (-)-norepinephrine. Values are means of duplicate or quadruplicate determinations from 2 experiments.

(B) Activation of frog erythrocyte membrane adenylate cyclase by (-)-isoproterenol and (-)-norepinephrine. Values are the means of duplicate determinations for 3 experiments.

---

forms. Finally, the presence of an unsaturated bond in this compound permits ready tritiation to high specific activities by catalytic reduction. Previously we have attempted to perform binding studies with [$^3$H](-)-propranolol in cardiac membranes (26). However, the presence of a very large number of low affinity, non-stereospecific sites possibly related to the marked "membrane" effects of this agent obscured identification of any high affinity binding sites.

A number of laboratories have encountered similar difficulties in attempting to bind radioactively labelled antagonists such as [$^3$H]propranolol (27), [$^3$H]phenoxybenzamine (28) and [$^3$H]-N-(2-bromoethyl)-N-ethyl-N-1-naphthyl-methylamine (29) to adrenergic receptors in various intact tissue preparations. Our studies with isolated membrane preparations now suggest that this is a more feasible approach. The catecholamine responsive adenylate cyclase provides an excellent biochemical response which, as demonstrated, directly parallels the data obtained by [$^3$H](-)-alprenolol binding.

The [$^3$H](-)-alprenolol binding sites identified in this study appear to have several of the characteristics one might expect of β-adrenergic receptor binding sites. First, the binding sites demonstrate strict stereospecificity. (-)-Isomers of the β-adrenergic antagonists are 50-100 fold more potent than the (+)-isomers, both in blocking [$^3$H](-)-alprenolol binding and in inhibiting stimulation of adenylate cyclase by isoproterenol (Table 1). (It has not been determined whether the activity exhibited by the (+)-isomers is due to contamination with the (-)-isomer.) Second, the binding sites show high affinity for

β-adrenergic antagonists ($K_D$ = $10^{-8}$ - $10^{-9}$M), and the inhibition of [$^3$H](-)-alprenolol binding by these antagonists correlates directly with their ability to antagonize isoproterenol stimulation of adenylate cyclase. The small differences between $K_D$'s for inhibition of [$^3$H](-)-alprenolol binding and inhibition of adenylate cyclase activation are possibly due to slight differences in experimental conditions. Third, the affinity of the [$^3$H](-)-alprenolol binding sites for β-adrenergic agonists closely parallels the potency of the agonists in stimulating adenylate cyclase. Finally, an α-adrenergic antagonist was inert in both inhibition of [$^3$H](-)-alprenolol binding and inhibition of isoproterenol stimulation of adenylate cyclase.

These binding data strongly suggest that [$^3$H](-)-alprenolol binding sites may indeed be equivalent to the adenylate cyclase-coupled β-adrenergic receptors. Definitive proof will require extensive comparison of binding and adenylate cyclase data for numerous agonists and antagonists in membranes from several tissues.

ACKNOWLEDGEMENTS. The authors wish to thank Drs. Ablad and Borg for the generous gift of (+)- and (-)-alprenolol and for helpful advice, and Ms. Catherine Bradley (New England Nuclear) for tritiation of the (-)-alprenolol. This work was supported by H. E. W. grant #HL-16037-01 and a grant-in-aid for the American Heart Association with funds contributed in part by the North Carolina Heart Association. Dr. Lefkowitz is an Established Investigator of the American Heart Association. Dr. Caron holds a fellowship from the Medical Research Council of Canada and the government of the province of Quebec.

REFERENCES.

1. Roth, J. (1973) Metabolism 22, 1059-1073.
2. Kahn, C. R. (1974) in Methods in Membrane Biology (E. D. Korn, ed.), vol. 3, in press, Plenum Press, N.Y.
3. Hall, Z. W. (1972) Ann. Rev. Biochem. 41, 925-952.
4. Freychet, P., Roth, J. and Neville, D. M. Jr. (1971) Biochem. Biophys. Res. Commun. 43, 400-408.
5. Changeux, J. P., Kasai, M. and Lee, C. Y. (1970) Proc. Nat. Acad. Sci. USA 67, 1241-1247.
6. Bockaert, J., Roy, C., Rajerison, R. and Jard, S. (1973) J. Biol. Chem. 248, 5922-5931.
7. Schramm, M., Feinstein, H., Naim, E., Long, M. and Lasser, M. (1972) Proc. Nat. Acad. Sci. USA 69, 523-527.
8. Marinetti, G. V., Ray, T. K. and Tomasi, V. (1969) Biochem. Biophys. Res. Commun. 36, 185-193.
9. Lefkowitz, R. J. and Haber, E. (1971) Proc. Nat. Acad. Sci. USA 68, 1773-1777.
10. Lefkowitz, R. J., Sharp, G. and Haber, E. (1973) J. Biol. Chem. 248, 342-349.
11. Lefkowitz, R. J., O'Hara, D. and Warshaw, J. (1974) Biochim. Biophys. Acta 332, 317-328.
12. Lefkowitz, R. J., O'Hara, D. and Haber, E. (1972) Proc. Nat. Acad. Sci. USA 69, 2828-2832.
13. Bilezikian, J. P. and Aurbach, G. D. (1973) J. Biol. Chem. 248, 5575-5583.
14. Bilezikian, J. P. and Aurbach, G. D. (1973) J. Biol. Chem. 248, 5584-5591.
15. Lefkowitz, R. J. (1974) Biochem. Pharmacol., in press.
16. Lefkowitz, R. J. (1974) in Frontiers in Catecholamine Research (E. Usdin and S. Snyder, eds.) pp. 361-368, Pergamon Press, N.Y.
17. Ablad, B., Brogard, M. and Ek, L. (1967) Acta Pharmacol. Toxicol. 25: Suppl 2, 9-40.

18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
19. Krishna, G., Weiss, B. and Brodie, B. B. (1968) J. Pharmacol. and Exptl. Therap. 163, 379 - 388.
20. Lefkowitz, R. J. (1974) J. Biol. Chem., in press.
21. Ramachandran, J. (1971) Anal. Biochem. 43, 227-239.
22. Furchgott, R. F. (1967) Ann. N.Y. Acad. Sci. 139, 553-570.
23. Rosen, O. M. and Rosen, S. M. (1968) Biochem. Biophys. Res. Commun. 31, 82-88.
24. Rosen, O. M. and Rosen, S. M. (1969) Arch. Biochem. and Biophys. 131, 449-456.
25. Lefkowitz, R. J. (1974) Biochem. Pharmacol., in press.
26. Vatner, D. and Lefkowitz, R. J. (1974) Mol. Pharmacol. 10, 450-456.
27. Potter, L. T. (1967) J. Pharmacol. and Exptl. Therap. 155, 91-100.
28. Yong, M. S. and Nickerson, M. (1973) J. Pharmacol. and Exptl. Therap. 186, 100-108.
29. Moran, J. F., May, M., Kimelberg, H. and Triggle, D. J. (1967) Mol. Pharmacol. 3, 15-27.