Anti-Alprenolol Antibodies in the Rabbit
A New Probe for the Study of β-Adrenergic Receptor Interaction

STANLEY G. ROCKSON, CHARLES J. HOMCY, AND EDGAR HABER

SUMMARY We immunized rabbits with an antigen prepared by covalent linkage of alprenolol, a β-adrenergic receptor antagonist, to bovine serum albumin. Competitive inhibition of [3H]dihydroalprenolol binding to antisera with a variety of unlabeled ligands revealed broad antibody specificity for β-adrenergic antagonists and agonists. The antiserum was subjected to affinity fractionation on hydroxybenzylpindolol-Sepharose 4B. Successive elution with 100 mM Tris HC1, 1 M NaCl, 4 M LiBr, and 5 M guanidine yielded fractions with increasing affinity for hydroxybenzylpindolol. The ligand-binding properties of these affinity-fractionated antibodies suggest that certain of these fractions recognize structural aspects of individual β-adrenergic ligands which are irrelevant to their biological activity, whereas others can be used to distinguish shared functional properties, such as the ethanolamine side chain, within the structural heterogeneity of β-adrenergic drugs. In particular, elution of hydroxybenzylpindolol-adsorbed antibody with (−)-propranolol allowed identification of an antibody fraction specific for the (−)-stereoisomer. Thus, affinity fractionation of antibodies raised against β-adrenergic ligands can provide useful analogues for the further study of the recognition properties of the β-adrenergic receptor.


IT IS now accepted that binding of pharmacologically active agents to a membrane receptor is the requisite first step in β-adrenergic modulation of cellular processes. Interaction of catecholamines with the β-adrenergic receptor initiates a cascade of biochemical events within which activation of membrane-associated adenylate cyclase mediates the target response. Recent attempts at characterization of the β-adrenergic system have used techniques for the in vitro quantification of receptor density and ligand-binding affinity of particulate membrane preparations (Levitzki et al., 1974; Alexander et al., 1975; Maguire et al., 1976), yet little is known about the molecular interaction of these drugs with their membrane-binding site. The biological activity of any β-agonist, through its augmentation of cyclic 3’5’-AMP production, or of a β-antagonist, which mediates the reversible inhibition of adenylate cyclase stimulation, is predicated on the structural specificity of the drug and is ultimately determined by the precise molecular configuration of the receptor-binding site. Recognizing that the structural characterization of β-adrenergic binding specificity must await successful purification of the receptor protein, we have pursued an alternate, indirect approach to structure-function relationships in the initial recognition step of β-adrenergic modulation of adenylate cyclase through the use of antagonist-specific antibodies.

Methods

Materials

(−)-[3H]Dihydroalprenolol (specific activity 58 Ci/mmol) was obtained from New England Nuclear. Stereoisomers of propranolol were a gift from Ayerst Laboratories; (+)-alprenolol from Astra Pharmaceuticals; and (+)-hydroxybenzylpindolol from Sandoz Pharmaceuticals. (+)-Propranolol was obtained from Sigma; aminopropyl-Sepharose 4B from BioRad; and Aquasol-2 from New England Nuclear. Goat antirabbit IgG was generously supplied by Dr. V. Zurawski.

Preparation of the Antigen

Covalent linkage of (±)-alprenolol to bovine serum albumin (BSA) yielded an antigen with a β-adrenergic antagonist as the hapten. Bromination of the olefin moiety of alprenolol was accomplished by the method of Vauquelin et al. (1977). Conversion of alprenolol to the bromohydrin derivative was determined by loss of the C=C signal as detected by NMR. Further proof was provided by quantitative analysis of the product, performed by Galbraith Laboratories. An equivalent weight of N-acetylimocysteine thiolactone was reacted with BSA to yield the thiolated derivative of the protein. The reactants were sealed under N2 for 12 hours at 25°C in Na2CO3 buffer, pH 8.5. The thiolated albumin was precipitated and washed in 0.1 M acetic acid, pH 3.5. After resuspension in 0.3 M Tris-2 mM EDTA, pH 8.2, the protein was reacted with bro-
moalprenolol, 1 mg/ml, for 4 days and dialyzed, successively, against 0.2 M sodium acetate, pH 4.5, and 0.1 M Tris-HCl, 1 mM dithiothreitol, pH 7.4. After lyophilization, the alprenolol-BSA conjugate was brought to a final concentration of 1 mg/ml in Tris buffer. Covalent linkage of (±)-alprenolol to BSA produced substitution of at least 1 mol alprenol/mol protein.

**Immunization Schedule**

Albino rabbits were immunized with the alprenolol conjugate at monthly intervals. One-half milliliter of the antigen preparation was emulsified in an equal volume of complete Freund's adjuvant and injected intradermally into the toepad. Ten-milliliter blood samples were obtained before immunization and at weekly intervals thereafter.

**Anti-Alprenolol Antibody Binding Assay**

The ligand affinities of the rabbit anti-alprenolol antisera were assessed with a double antibody precipitation, competitive binding assay. The assays were performed in identical Sarstedt plastic tubes. Each tube received a 100-μl aliquot of immune serum, diluted 1:10 in phosphate-buffered saline; 50 μl of (±)[3H]alprenolol, 5–100 nM; and 50 μl of the unlabeled β-adrenergic ligand of interest. Total binding of the label to antiserum was determined in the absence of added, unlabeled ligand; a 1:10 dilution of preimmune serum served as the control for anti-alprenolol binding. After a 1-hour incubation at 25°C, 100 μl of goat antirabbit IgG was added to each tube with 50 μl of normal rabbit IgG added as a carrier for the precipitation. The tubes were again incubated at 25°C for 30 minutes. Immune precipitates were washed twice in a Sorvall tabletop centrifuge at 2000 rpm for 10 minutes and solubilized in 1 ml of 1% sodium dodecyl sulfate. The samples were counted in the presence of 10 ml of Aquasol-2 in a Packard Tricarb liquid scintillation counter (tritium counting efficiency is 35%). At each concentration of unlabeled ligand in this assay, the results are expressed as a percentage of total binding capacity of titiated label to the antiserum. All measurements of bound radioactivity are corrected for nonspecific binding to the preimmune serum sample, which averaged 10%. Under these assay conditions, saturation of the antibody varies between 0 and 100%, depending on the ambient concentration of free ligand. The concentration of labeled ligand present is always adequate to produce full saturation because, in the absence of unlabeled ligand, only 5% of the total radioligand is bound.

**Affinity Fractionation of Anti-Alprenolol Antisera**

A hydroxybenzylpindolol-substituted resin was prepared using a diazonium derivative of agarose (Cuatrecasas, 1970), prepared in the following manner. Ten milliliters of aminopropyl-Sepharose were eventually equilibrated in 100% dimethylformamide by washing the resin with solutions containing an increasing ratio of dimethylformamide to water. Two milliliters of pyridine and 2 g of p-nitrobenzolazide were added, and the reaction was allowed to proceed for 48 hours at 4°C. Completeness of the substitution was determined by the loss of reactivity with fluorescamine. The resin was washed extensively with dimethylformamide, then reequilibrated in an aqueous buffer and reduced with 0.1 M Na dithionite in 0.5 M NaHCO₃, pH 8.5. Successful reduction of the -NO₂ group was determined by the reappearance of fluorescamine reactivity. The diazonium derivative of the resin was obtained by reaction with 0.1 M NaNO₂ in 0.5 N HCl. Ten milliliters of the gel immediately were reacted with 100 mg of (±)-hydroxybenzylpindolol to form the diazo conjugate.

**Results**

Immunization of rabbits with (±)-alprenolol-BSA elicited the production of an antiserum, the component antibodies of which were directed against the common features of several structurally distinct β-adrenergic ligands. Examination of the binding affinities of the antibody (Fig. 1) reveals a high affinity interaction not only with the hapten, alprenolol, but also with the structurally distinct β-antagonists, propranolol and hydroxybenzylpindolol. Competitive displacement of the bound 3H(–)-dihydroalprenolol occurs at concentrations of unlabeled ligand which compare favorably to the binding affinity of the in vitro β-adrenergic membrane receptor for these antagonists (U'Prichard et
binding capacity for $^3$H(--)-dihydroalprenolol by with each buffer was determined by the absence of
approximate binding occurred at approximately $10^{-7}$ M ($\pm$)-alprenolol, the displacement by this ligand appeared to be biphasic, indicative of a second, high connectivity of binding sites for alprenolol.

In addition to the observed high affinity interaction with these structurally diverse $\beta$-agonists, the anti-alprenolol antibody also specifically binds the structurally more remote $\beta$-agonists (Fig. 1). Specific displacement of $^3$H(--)-dihydroalprenolol occurs with norepinephrine and isoproterenol ($5 \times 10^{-7}$ M) but not with the biologically inactive analogue, pyrocatechol (Fig. 1). Although the native antiserum does not precisely fulfill the established binding potency criteria for the $\beta$-adrenergic receptor, the absolute magnitude and relative affinities of this population of antibody-binding sites display distinct similarities to the ligand recognition properties of the cellular receptor. Thus, in analogy to the membrane $\beta$-adrenergic receptor, the affinity of these antibody sites for certain of the $\beta$-agonists exceeds the affinity for the potent agonists tested by at least two orders of magnitude.

Analysis of binding equilibrium of $^3$H(--)-dihydroalprenolol to the antibody produced a multiphasic Scatchard curve, indicative of heterogeneous affinity of the antiserum for this antigen. Since the immune response to a haptenic antigen normally comprises a broad class of antibodies with varying binding affinity and specificity (Eisen and Siskind, 1964; Haber, 1968), affinity fractionation of this anti-alprenolol antiserum might be expected to yield binding site subpopulations which would identify distinct structure-function relationships. To this end, we chose to substitute an agarose affinity resin with a binding ligand structurally dissimilar to the original hapten. Hydroxybenzylpindolol differs substantially from alprenolol, in that an indole group is substituted for the benzene ring, and the ethanolamine side chain contains an additional phenol substituent.

Ten milliliters of anti-alprenolol antiserum were passed over a 5-ml (±)-hydroxybenzylpindolol-Sepharose resin and eluted with 100 mM Tris buffer. The fall-through, nonadsorbed antibody fraction was collected and pooled for competitive binding assay. To elute fractions with increasing affinity for the resin substituent, the column was developed successively with 1 M NaCl, 4 M LiBr, and 5 M guanidine HCl (Fig. 2). The completeness of elution with each buffer was determined by the absence of desorbed protein in the effluent, measured by absorbance at 280 nm. Those fractions that displayed binding capacity for $^3$H(--)-dihydroalprenolol by double antibody precipitation were pooled for analysis. The LiBr fraction was diluted 1:10 with buffer prior to assay, and the guanidine fraction was tested after extensive dialysis against 10 mM Na acetate, pH 5.5.

Fractionation of the antibody pool by affinity adsorption yields subsets of antibodies with wide variations in the affinity for the $\beta$-adrenergic ligands of interest (Fig. 3, A–C). The fall-through fraction retained weak affinity for (±)-alprenolol and (±)-propranolol but failed to bind (±)-hydroxybenzylpindolol, as predicted by its behavior on the affinity resin. In addition, this fraction failed to bind the agonist, (±)-isoproterenol, in the range of biological interest. The column-retarded fractions, eluted with Tris buffer, demonstrated a moderate affinity for all $\beta$-adrenergic ligands tested. In contrast, the LiBr fraction demonstrated substantial affinity for alprenolol, yet failed to interact appreciably with the structurally similar $\beta$-antagonist, (±)-propranolol; competitive inhibition with unlabelled (±)-hydroxybenzylpindolol revealed significant binding of this structurally dissimilar antagonist. Finally, the guanidine effluent demonstrated the expected high affinity for (±)-hydroxybenzylpindolol, the resin substituent, and bound the other antagonists with lesser affinity.

A prominent characteristic of $\beta$-adrenergic ligands is the stereospecific requirement for their biological activity. The potential stereospecific recognition of antibody binding sites has been documented for the optically resolved isomers of tartaric
anti-alprenolol antibodies

Figure 3

Binding profiles of anti-alprenolol antibody fractions prepared by hydroxybenzylpindolol affinity fractionation. The fall-through (■), column-retarded (○), LiBr-eluted (□), and guanidine-eluted (△) fractions were pooled for assay. Competitive inhibition of [3H]—dihydroalprenolol binding by unlabeled (±)-propranolol (panel A), (±)-alprenolol (panel B), and (±)-hydroxybenzylpindolol (panel C) was assessed for each fraction. In panel D, the competitive inhibition of unlabeled (±)-isoproterenol to the fall-through and column-retarded fractions is compared with the binding profile of the unfractionated antiserum (▲).

Discussion

Recent interest in the interaction of small molecules with biological membranes has resulted in the identification of specialized, high affinity binding sites on cellular membranes. Requisite properties of cellular receptors include reversible, high affinity binding, reflecting the biological potency of the native ligand and its structural analogues (Haber and Wrenn, 1976; Cuatrecasas, 1974). The β-adrenergic binding site of frog erythrocytes (Mukherjee et al., 1975), turkey erythrocytes (Levitzki et al., 1974; Brown et al., 1976), and canine myocardium (Alexander et al., 1975; Wrenn and Haber, 1979) have been characterized with respect to ligand-binding properties, yet intensive investigation of the structural properties of the binding site has been hampered by the difficulties inherent in the quantification of binding to membrane preparations or detergent-solubilized proteins (Maguire et al., 1976).

An essential characteristic for the identification of a β-adrenergic receptor is the requirement for the stereospecificity of ligand recognition. In certain circumstances, this potential may also be expressed by antibody-combining sites (Landsteiner, 1945). Immunization with an enantiomeric antigen may elicit the production of stereospecific combining sites.

Several of these requisite characteristics of a true receptor-binding site are fortuitously shared by the antigen-combining site of immunoglobulins. The response to antigenic stimulation consists of the biosynthesis of proteins (antibodies) that possess high inherent specificity and affinity for the immunogen (Haber, 1968). Thus, it might be predicted that alprenolol, used as an antigenic determinant, will result in the production of stereospecific combining sites.

Unfractionated alprenolol antiserum, as previ-
ously reported (Hoebeke et al., 1978), demonstrates a binding affinity for alprenolol analogous to the β-adrenergic receptor. We now have shown that the relative binding potency of several β-adrenergic antagonists and agonists parallels the in vivo potency for adenylate cyclase modulation. Fractionation of the pooled antibody by affinity chromatography affords additional insight into the functional relationship of structural diversity to binding. Elution of adsorbed antigen into fractions of increasing affinity to a second ligand, hydroxybenzylpindolol, identifies binding site subsets with different specificities for the shared class of antagonists and agonists. Finally, elution from the column with (–)-propranolol permits fractionation into a class of binding sites with preferential binding of the (–) enantiomer, thus fulfilling the property of stereospecificity for this antibody analogue of the physiological receptor.

Recently, Caron et al. (1979) have reported β-adrenergic ligand-binding activity in rabbit antisera raised against the partially purified β-adrenergic receptor of frog erythrocytes. The implications of that study differ substantially from the present characterization of anti-alprenolol antibodies. In that investigation, the detection of β-adrenergic ligand-binding activity of rabbit antiserum arose fortuitously from an attempt to generate antireceptor antibodies. Although the identity of the actual immunogen is unclear, the authors speculate that the partially purified receptor serves as carrier for antibody. Furthermore, the heterogeneous binding data indicated the anticipated heterogeneous nature of the immune response, even to a haptens of limited antigenic diversity (Haber, 1968). The Scatchard plot of our binding data indicated the anticipated heterogeneity of dissociation constants expected in an hapten-specific antibody. Furthermore, the heterogeneous nature of the rabbit immune response to (±)-alprenolol, a catecholamine analogue, is underscored by our attempts at affinity fractionation of the antiserum, which yielded partial resolution into subpopulations of antigen-combining sites with diverse binding specificities.

Thus, we have demonstrated that the β-adrenergic antagonist, (±)-alprenolol, can serve as a potent antigenic determinant when covalently bound to a protein carrier. The immune response to (±)-alprenolol comprises a population of binding sites with broad specificity for β-adrenergic ligands. Affinity fractionation of antiserum has been used to identify classes of binding sites directed against the shared functional properties of these structurally diverse drugs. In particular, elution with (–)-propranolol can serve to identify a fraction of binding sites with the appropriate specificity for biologically active enantiomers of these drugs. It appears that these techniques are useful for the identification and characterization of antibody analogues with some of the binding properties of the β-adrenergic receptor. In addition to the continued, direct study of affinity-fractionated antibody classes from immune rabbit sera, application of newer cell hybridization techniques (Lembcke et al., 1978; Melchers et al., 1978) to these investigations should yield valuable additional insights into the nature of β-adrenergic binding. Thus, the tissue culture separation of hybridized immunocompetent cells provides the potential for identification of monoclonal antibodies with distinct β-adrenergic binding properties. Isolation of the individual antibody molecules of interest will permit intensive investigation into the molecular nature of β-adrenergic ligand binding to the antibody recognition site. Furthermore, monoclonal antibodies, as well as affinity-fractionated rabbit antiserum, can be used as antigens for the production of antidiotype antibodies which can be predicted to interact with a similarly structured binding site on the β-adrenergic receptor itself. Such study of antidiotype antibodies has already been proven to be of use in the identification of insulin-binding sites on cell membranes (Sege and Peterson, 1978). Because of the demonstrated use of these techniques for insulin, a structurally complex polypeptide hormone, the probability is great that alprenolol, a binding ligand of limited structural diversity, will yield antidiotype antibodies with high affinity for the biological receptor.

References
Eisen HH, Siakind GW (1964) Variation in affinities of antibodies during the immune response. Biochemistry 3: 996–1008


Anti-alprenolol antibodies in the rabbit. A new probe for the study of beta-adrenergic receptor interaction.
S G Rockson, C J Homcy and E Haber

Circ Res. 1980;46:808-813
doi: 10.1161/01.RES.46.6.808

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1980 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/46/6/808

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/