Affinity Chromatography of the \( \beta \)-Adrenergic Receptor*

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Procedures are described for synthesis of a new alprenolol-agarose derivative which is uniquely useful for purification of solubilized \( \beta \)-adrenergic receptors. Preparation of the gel is simple and reproducible. Introduction of a hydrophilic spacer arm with the use of a long chain bisoxirane reagent has minimized nonspecific adsorption of protein to the gel. Using a digitonin-solubilized preparation of frog erythrocyte \( \beta \)-adrenergic receptors we have validated the biospecific nature of adsorption and elution of receptors from the alprenolol gel. Adrenergic agents block adsorption of receptors to the gel and elute receptors from the gel with a typical \( \beta \)-adrenergic specificity. Among agonists the order of potency for both processes is isoproterenol \((EC_{50} = 4 \text{ to } 7 \mu M)\) > epinephrine > norepinephrine. Dopamine and carbachol are ineffective. Adsorption and elution of receptors display marked stereospecificity with \((+)-\)isoproterenol being 100 times more potent than \((-)-\)isoproterenol. Among antagonists \((+)-\)alprenolol \((EC_{50} = 30 \text{ to } 45 \text{ nm})\) is 30 times more potent than \((-)-\)alprenolol. The \( \alpha \)-adrenergic, dopaminergic, and cholinergic antagonists phentolamine, haloperidol, and atropine are ineffective.

In batch or column experiments, up to 95% of receptors in a soluble preparation could be adsorbed to the gel and specifically eluted with up to a 60% yield. A single cycle of affinity chromatography resulted in a 100- to 200-fold purification and two cycles yielded 1200- to 1500-fold purification. Preparations cycled twice through the affinity support had a specific activity of 1900 pmol/mg of protein of \[^{3}H\]dihydroalprenolol binding at saturating ligand concentrations. These preparations were purified over 18,000-fold from receptor preparations in crude lysate preparations. The binding characteristics of these highly purified preparations appeared to be identical to those of the unpurified soluble preparations.

Results and Discussion

In this paper we describe an affinity chromatography procedure by which a substantial purification of the soluble frog erythrocyte \( \beta \)-adrenergic receptor can be achieved. A soluble preparation of the \( \beta \)-adrenergic receptor obtained by treatment of purified frog erythrocyte membranes (6) with 1% digitonin was chromatographed on Sepharose 4B-alprenolol. Fig. 1 shows a typical elution profile when 60 ml of this preparation was chromatographed on 10 ml of the alprenolol gel. While about 90% of the total receptor activity was specifically bound, more than 90% of the total protein was unretarded by the column. Following washing of the column with

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to the gel or in eluting activity from the alprenolol column. The effects of isoproterenol and alprenolol were shown to be miniprint supplement). isomers over the (+)-isomers of these drugs (cf. Figs. 3 to 6). Results indicate that the use of a long chain bisoxirane reagent (1,4-butanediol diglycidylether) to introduce a hydrophilic spacer arm on Sepharose-4B (7,8) and covalently immobilize alprenolol (9) appears to minimize non-specific adsorption of protein and receptor activity to the gel.

Indeed, the interaction of the soluble receptors with the immobilized ligand appears to be solely on the basis of biospecific affinity interactions. Preincubating the soluble preparations with various agents it was possible to demonstrate that adrenergic agonists and antagonists blocked retention of the receptors by the alprenolol gel with characteristics identical to those of a β-adrenergic process. Elution of receptor activity from the gel was also shown to reflect this typical β-adrenergic specificity (cf. Figs. 3 to 6, miniprint supplement). Thus, agonists blocked retention by and eluted receptor activity from the alprenolol gel with the potency order (−)-isoproterenol > (−)-epinephrine > (−)-norepinephrine, whereas dopamine and carbachol were ineffective. Blockade of retention and elution of receptor activity was also caused by the β-adrenergic antagonist (−)-alprenolol, whereas α-adrenergic, dopaminergic, and cholinergic antagonists were ineffective. The effects of isoproterenol and alprenolol were shown to be dose-dependent and, furthermore, these processes displayed stereoselectivity of 100- to 90-fold, respectively, for the (−)-isomers over the (+)-isomers of these drugs (cf. Figs. 3 to 6 miniprint supplement).

One rather unexpected finding was the high potencies of both agonists and antagonists in inhibiting receptor binding to the gel or in eluting activity from the alprenolol column. (−)-Alprenolol and (−)-isoproterenol inhibited retention and eluted receptor activity with $E_{\text{C50}}$ values of 30 to 45 nM and 4 to 7 μM, respectively, which is in the same range as the $E_{\text{C50}}$ values obtained when these drugs are competing for about 10 nM [3H]dihydroalprenolol in a binding assay. Yet, the concentration of alprenolol on the gel as measured by elemental analysis is in the millimolar range. Therefore, it may be that some of the substituted alprenolol on the agarose is not accessible to the soluble receptor or that the affinity of the immobilized ligand has been reduced considerably, or both.

Table I shows a typical purification scheme of a soluble receptor preparation that has been chromatographed successively through two Sepharose 4B-alprenolol gel steps. A first batchwise fractionation of the soluble receptor preparation on the alprenolol gel yields an overall purification of about 100- fold with a recovery of 35%. Subsequent chromatography of the material specifically eluted in this batch step on an alprenolol gel column produced a further 10- to 15-fold increase in specific activity with a recovery of 40 to 50%, representing a 10 to 15% overall recovery of the initial material. As shown in the table the specific activity of pooled fractions across the peak of activity eluted from the affinity column varied from 900 to 1500 pmol/mg with an average of about 1200 pmol/mg. This represents a 1200- to 1500-fold purification over the starting soluble preparation or a 10,000- to 15,000-fold enrichment in specific activity of binding over that present in crude erythrocyte membranes (100 fmol/mg of protein). It should be noted that since binding activity in these studies has been routinely assayed at about 75% of saturation the value of 1200 pmol/mg is readily of the order of 1600 to 2000 pmol/mg.

As we have shown (Fig. 7, in miniprint), the β-adrenergic receptor which has been partially purified through one or two cycles over the affinity gel retains all the essential specificity and affinity characteristics of the starting soluble receptor. Competition curves for several agonists and antagonists for binding of [3H]dihydroalprenolol were virtually identical in all three preparations.

It is also of interest to mention here that chromatography of a solubilized erythrocyte membrane preparation on the affinity column yields a partially purified β-adrenergic receptor preparation which is devoid of any measurable adenylate...
A digitonin-soluble receptor preparation was first fractionated on the affinity gel in a batchwise fashion. One hundred fifty milliliters in 1% digitonin, 0.05 mM dithiothreitol, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, was incubated overnight (10 h) at 23°C with 25 to 30 ml of alprenolol gel pre-equilibrated with 0.2% digitonin, 0.05 mM dithiothreitol, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, with slow rotation of the flask. The supernatant was removed and the gel was washed five times with 50 to 70 ml fractions of cold equilibrium buffer. Receptor binding activity was eluted by incubation of the gel at 23°C for 3 h with 1 mM (±)-isoproterenol in the equilibrium buffer. Material obtained from two batch was pooled, lyophilized, chromatographed on Sephadex G-50, loaded over a 10-ml alprenolol gel column, and chromatographed as described in the legend to Fig. 1. [3H]Dihydraloprenol binding activity for all fractions was assayed arbitrarily at 16 nm and, therefore, represents about 75% of the value for saturation binding in all fractions. Data shown for the second cycle of affinity gel are values obtained on four pooled fractions taken across the peak of activity eluted from the affinity column. Values shown for recovery and total binding of this material are for the total purified material pooled from these four regions of the peak.

In conclusion, the β-adrenergic receptor affinity chromatography procedures described here should be easily and reliably applicable to large scale purification of the receptor protein from various sources.

REFERENCES


Additional references are found on p. 2927.
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EXPERIMENTAL PROCEDURES

SERUM: 1.1 mg of β-adrenergic blocking agent was prepared according to the procedure described in the Methods section. Briefly, 1.1 mg of the blocking agent was dissolved in 1 ml of 0.1 M sodium phosphate buffer (pH 7.4) and mixed with 2 ml of 0.1 M sodium azide. The solution was then filtered through a Millipore filter (pore size 0.22 μm) and stored at −20°C. 

PLASMA: 1.1 mg of blocking agent was dissolved in 1 ml of 0.1 M sodium phosphate buffer (pH 7.4) and mixed with 2 ml of 0.1 M sodium azide. The solution was then filtered through a Millipore filter (pore size 0.22 μm) and stored at −20°C.

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ILLUMINATION: The blocking agent was then incubated with plasma samples at room temperature for 30 min. After incubation, the solution was filtered through a Millipore filter (pore size 0.22 μm) and stored at −20°C.

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RESULTS AND DISCUSSION

The results of the experiment are shown in Table 1. The blocking agent was found to be effective in inhibiting the binding of serum and plasma samples to the γ-H3-adrenergic receptor. The inhibition was maximal at a blocking agent concentration of 0.1 M and decreased to 50% at 0.01 M. The results also indicated that the blocking agent was effective in inhibiting the binding of plasma samples to the γ-H3-adrenergic receptor. The inhibition was maximal at a blocking agent concentration of 0.1 M and decreased to 50% at 0.01 M. The results also indicated that the blocking agent was effective in inhibiting the binding of plasma samples to the γ-H3-adrenergic receptor. The inhibition was maximal at a blocking agent concentration of 0.1 M and decreased to 50% at 0.01 M.

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was routinely used in these experiments to prevent desorption of catecholamines (Fig. 5). As shown in Fig. 5, elution of the receptor activity was achieved by catecholamines in the following order of effectiveness: 1, dichloromethane, 2, ethyl acetate, 3, methanol, 4, ethanol, 5, acetonitrile. Each compound also eluted receptor activity in the same order at the molar equivalent mol/L. However, the degree of desorption was found to vary inversely with the flow rate at a fixed flow rate of 0.1 mL/min. All desorption to columns were routinely performed at room temperature since chromatography in the cold (6°C) seemed to reduce the efficiency of desorption.

Large scale columns were routinely washed in the cold, since this was found to slow the desorption of the receptor from the alumina. Support: Tego, a small amount of receptor activity was present in warm buffer at room temperature but this was considered a result of the washing of the columns with buffer and not due to steric hindrance of the receptor with the alumina. All desorption of the receptor was performed with 50% ethanol at 6°C to maintain the same buffer concentration as in the cold. Experiments with three different batches of alumina showed no change in the activity bound to the gel. In experiments with gel derived from the same batch, the extent of the recovery was very reproducible. These experiments represent the optimal conditions for recognition and recovery of receptor activity.

4-5 M solubilized receptor preparation in 5 mL alumina gel column yields 95% or more desorption given a flow rate of 0.1 mL/min. Indeed, the extent of desorption was found to vary inversely with the flow rate at a fixed flow rate of 0.1 mL/min. All desorption to columns were routinely performed at room temperature since chromatography in the cold (6°C) seemed to reduce the efficiency of desorption.

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As with the binding, elution of receptor activity from the column by either beta-adrenergic agonist or antagonist also displayed characteristic differences. As shown in Fig. 5B, the beta-agonist eluted receptor activity from the gel in a manner similar to the beta-agonist eluted receptor activity from the gel in a manner similar to the beta-agonist eluted receptor activity from the gel in a manner similar to the beta-agonist eluted receptor activity from the gel in a manner similar to the beta-agonist eluted receptor activity from the gel in a manner similar to the beta-agonist eluted receptor activity from the gel in a manner similar to the beta-agonist eluted receptor activity from the gel in a manner similar to the beta-agonist eluted receptor activity from the gel in a manner similar to the beta-agonist eluted receptor activity.

Properties of the chromatography of soluble receptor preparations on Sepharose 4B alumina gel. It was found that adsorption of receptor activity to the support was dependent upon the ratio of gel to preparation used. For example, chromatography of a 40-60% diluted receptor preparation on a 5 mL alumina gel column yielded 95% or more desorption given a flow rate of 0.1 mL/min. Indeed, the extent of desorption was found to vary inversely with the flow rate at a fixed flow rate of 0.1 mL/min. All desorption to columns were routinely performed at room temperature since chromatography in the cold (6°C) seemed to reduce the efficiency of desorption.

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FIG. 5. ELUTION OF ADRENERGIC AGONISTS AND OTHER AGENTS TO BIODISTRIBUTIONALLY ELUTE BS-ADRENERGIC RECEPTOR ACTIVITY FROM SEPHADEX G-200 SIMPLE IN VIVO AND IN VITRO EXPERIMENTS. EXPERIMENTAL CONDITIONS WERE USED AS DESCRIBED IN THE METHODS. A. ALUMINA GEL FILLS WAS WASHED BY 5 M OF THE RECEPTOR ASH WITH A SOLUTION OF (1) DISSOLVED OXIDIZED BY 5 M OF THE ANTAGONISTS AT WASHING CONCENTRATIONS TO ELUTING THE RECEPTOR ACTIVITY. RESULTS SHOWN ARE REPRESENTATIVE OF 2-3 EXPERIMENTS.

FIG. 6. ELUTION OF ADRENERGIC ANTAGONISTS AND OTHER AGENTS TO BIODISTRIBUTIONALLY ELUTE BS-ADRENERGIC RECEPTOR ACTIVITY FROM THE SEPHADEX G-200 SIMPLE IN VIVO AND IN VITRO EXPERIMENTS. EXPERIMENTAL CONDITIONS WERE USED AS DESCRIBED IN THE METHODS. A. ALUMINA GEL FILLS WAS WASHED BY 5 M OF THE RECEPTOR ASH WITH A SOLUTION OF (1) DISSOLVED OXIDIZED BY 5 M OF THE ANTAGONISTS AT WASHING CONCENTRATIONS TO ELUTING THE RECEPTOR ACTIVITY. RESULTS SHOWN ARE REPRESENTATIVE OF 2-3 EXPERIMENTS.
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