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EVIDENCE FOR TYROSINE AT THE LIGAND BINDING CENTER OF BETA-ADRENERGIC RECEPTORS

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Abstract—Beta$_1$- and beta$_2$-adrenergic receptor radioligand antagonist binding activities in plasma membrane preparations from mammalian lung, as well as amphibian and avian red blood cells, have been shown to be inactivated by agents which specifically alkylate tyrosine. In membrane preparations, protection against inactivation was afforded by both agonists and antagonists. In soluble purified preparations, antagonists but not agonists protected against inactivation. These results suggest that tyrosine is located at or near the ligand binding site of both beta$_1$- and beta$_2$-adrenergic receptors.

In response to agonist binding site occupancy by epinephrine and norepinephrine, beta-adrenergic receptors stimulate the enzyme adenylate cyclase to produce cyclic AMP [1]. Modulation of enzyme activity has been shown to occur by activation of a GTP binding regulatory protein, by agonist occupied receptor [2, 3]. Receptors then contain at least two functional centers, one to recognize ligands and another to communicate with additional cyclase-linked components.

While considerable progress has been reported in the isolation and characterization of beta-adrenergic receptor proteins [4, 5], little is known of the amino acid content or topography of ligand binding or functional communication domains.

Information about protein active centers can be obtained by the use of selective amino acid or functional group alkylating or modifying agents [6, 7]. In the study described here, we have examined the effects on beta receptor antagonist ligand binding activity of agents which alkylate tyrosine. These studies provide chemical evidence for the presence of an aromatic amino acid, tyrosine, as an integral component of the agonist/antagonist binding site of beta-adrenergic receptors.

MATERIALS AND METHODS

Purified frog erythrocyte membranes were a gift from Dr. Marc Caron (Howard Hughs Medical Institute, Duke University, Durham, NC). Rat, rabbit, and guinea pig lungs were obtained from Hazelton Dutchland (PA). Fluorosulfonylnapthoic acid was provided by Dr. Kenneth Holden (SKF-Med. Chemistry). *p*-Nitrobenzylsulfonylfluoride was obtained from the Pierce Chemical Co. (Chicago, IL). All other materials and reagents were obtained as described [4].

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Preparation of plasma membranes. Purified frog erythrocyte plasma membranes were prepared in the presence of soybean trypsin inhibitor (10 μg/ml), benzamidine (10^{-4} M), EDTA (10^{-5} M), bacitracin (50 μg/ml), and phenylmethylsulfonylfluoride (10^{-5} M) as in Ref. 8. Purified turkey red blood cell (RBC) plasma membranes were prepared as in Ref. 3. Bovine, rat, guinea pig, and rabbit lung plasma membranes were prepared by homogenization (30% w/v; Waring blender) in degassed, ice-cold 75 mM Tris–HCl, pH 7.2, 25 mM $MgCl_2$ with 50 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10^{-4} M benzamidine, 50 μg/ml leupeptin, 5 μg/ml chymostatin and 10^{-5} M phenylmethylsulfonylfluoride. Homogenates were equilibrated (40 min) at 4° with 800–1000 psi of purified nitrogen in a Parr bomb and released to atmospheric pressure. After dilution (3-fold) with homogenization buffer and a low-speed centrifugation (2000 *g* for 20 min, 4°), plasma membranes were collected by centrifugation of supernatant fractions at 18,000 *g* for 60 min (4°). Pellets were washed with homogenization buffer and finally resuspended to 5–10 mg/ml protein before being frozen in liquid nitrogen and stored at −70° until use.

Preparation of purified beta-adrenergic receptor. Purified beta-adrenergic receptors were obtained from the plasma membranes of turkey red blood cells as in Refs. 4 and 9. Briefly, digitonin-solubilized receptor was affinity chromatographed using alprenolol-Sepharose. Bound receptors were eluted with alprenolol. Receptor ligand binding activity was concentrated by ultrafiltration using Amicon PM-30 membranes and desalted free of eluting ligand by Sephadex G-50 chromatography [4, 8, 9]. Turkey red blood cell plasma membrane beta-receptors are exclusively of the beta$_1$-adrenergic subtype [4].

Assays. Particulate preparations of frog and turkey red blood cells, and rat, rabbit, and bovine lung plasma membranes were assayed for beta-adrenergic receptor ligand binding activity with [^{125}I]cyanopindolol ([^{125}I]CYP) or [^{3}H]dihydroalprenolol ([^{3}H]DHA) as in Ref. 4. Bound ligands were sep-

arated from free ligands by vacuum filtration over Whatman Gf/C glass fiber filters, and bound radioactivity was determined using a Beckman GP-5500 gamma counter or an LS 7500 liquid counter with HP/b counting fluor. Protein concentrations were estimated by the method of Bradford [10] using bovine serum albumin as standard.

Solubilized receptor was assayed with [^{125}I]CYP or [^{3}H]DHA as in Refs. 4 and 8 with bound ligand separated from free ligand by Sephadex G-50 chromatography [4].

Reaction of particulate receptor preparations with alkylating agents. Membranes were equilibrated with 75 mM Tris–HCl, pH 8.0, 25 mM $MgCl_2$ by dilution and centrifugation. Alkylating agents were prepared as stock solutions from 0.025 to 0.1 M in isopropyl alcohol and diluted by direct addition to receptor membrane preparations as follows: to each tube was added approximately 1 mg of plasma membrane protein and 500 μl of 75 mM Tris–HCl, 25 mM $MgCl_2$ (pH 8) and alkylator to the appropriate final concentration. Controls received equal volumes of isopropyl alcohol only. After a 30-min reaction at room temperature, 50-μl samples of the reaction mixture were transferred to duplicate tubes containing [^{125}I]CYP (200 pM) in the presence or absence of 10^{-5} M alprenolol. Incubations with radioligand were for 1 hr at 25°, after which bound was separated from free radioligand by filtration as described above. Alternatively, alkylating agent was removed by repeated centrifugation and resuspension (Beckman Microfuge, 10 min at 25°) prior to assay.

Reaction of particulate receptor preparations with alkylating agents in the presence of adrenergic agents. Protection experiments were performed essentially as described above except that membranes (frog red blood cell) were preincubated with 10^{-4} M (±)alprenolol, (−)isoproterenol, (+)isoproterenol or buffer for 15 min at room temperature prior to the addition of alkylating agent. After an additional 30 min (25°), excess adrenergic agents and alkylator were removed by dilution to 1.5 ml with 75 mM Tris–HCl, pH 7.4, 25 mM $MgCl_2$, 10^{-4} M Gpp(NH)p and centrifugation in a Beckman microfuge. This procedure was repeated four times with final pellets

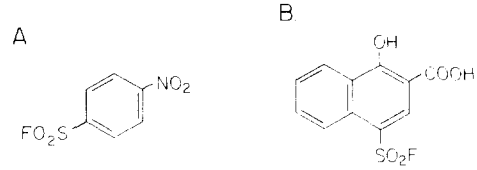

Fig. 1. Molecular structures of pNBSF (A) and FSNA (B).

resuspended in 100 μl of washing buffer. Receptor binding activity was measured by assay with [^{125}I]CYP of a 20-μl aliquot of each reaction condition.

Reaction of soluble purified receptors with alkylating agents. Purified soluble beta$_1$-adrenergic receptors were obtained from turkey red blood cell plasma membranes as described in Ref. 9. Time–course estimations of receptor inactivation were constructed by addition of fluorosulfonylnapthoic acid (10^{-3} M) to tubes containing 300–500 fmoles of ligand binding activity in the presence or absence of 10^{-5} M isoproterenol or 10^{-5} M alprenolol. After incubation for various times, aliquots were desalted, to remove the alkylator and adrenergic agent by Sephadex G-50 chromatography, and assayed with [^{125}I]CYP as described in Ref. 3. Similar experiments were performed with epinephrine and propranolol. All buffers contained 10^{-3} M digitonin, 100 mM NaCl, 10 mM Tris, pH 8.0.

RESULTS

Two reagents that alkylate tyrosine, *p*-nitrobenzylsulfonylfluoride (pNBSF) and fluorosulfonylnapthoic acid (FSNA) (Fig. 1), were examined for their effects on the abilities of particulate preparations of beta$_1$- and beta$_2$-adrenergic receptors to bind the radioligand antagonist [^{125}I]CYP. Frog RBC plasma membranes contain beta receptors exclusively of the beta$_2$ subtype [8]. As shown in Fig. 2A, for these preparations, both reagents inactivated [^{125}I]CYP binding in a dose-dependent fashion although FSNA appeared to be more potent. The effects of FSNA were also examined on plasma membrane preparations obtained from mammalian lung

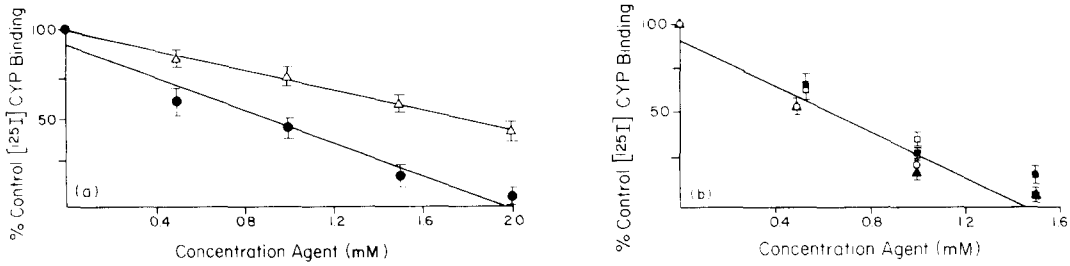

Fig. 2. Inactivation of beta-adrenergic receptor by tyrosine-directed agents. (A) Purified frog red blood cell plasma membranes were incubated with pNBSF (△) and FSNA (●) at the concentrations shown. After reaction, aliquots of each incubation mixture were assayed in duplicate with the antagonist [^{125}I]-CYP as in Refs. 4 and 8. Radioligand binding activity expressed on the ordinate axis is defined as percent of control incubations that received no alkylating agents. (B) Equal amounts of purified plasma membrane protein from frog red blood cell (○), rat lung (□), rabbit lung (▲), and guinea pig lung (■) were reacted with FSNA as in panel A, and assayed for receptor radioligand binding activity. Frog red blood cell = 100% beta$_2$, guinea pig lung and rat lung = 80% beta$_2$, and rabbit lung = 80% beta$_1$ [11]. For both panels, N = 2 where the distance between the bars is the range with the data point at the center of it.

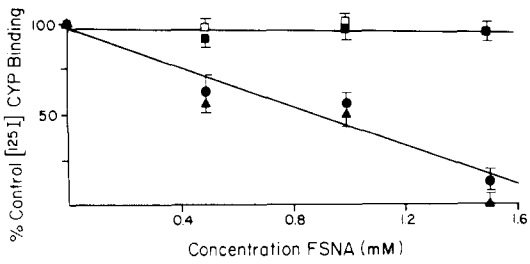

Fig. 3. Protection against fluorosulfonylfluoride inactivation by adrenergic agents. Purified frog red blood cell plasma membranes were incubated with 10^{-4} M (−)isoproterenol (□), (±)alprenolol (■), (+)isoproterenol (▲) or buffer (●), 15 min (25°) prior to addition of FSNA to the concentration shown and a further 30-min reaction. Alkylating and adrenergic agents were then removed by extensive washing as described in Materials and Methods, and aliquots of each incubation mixture were assayed in duplicate for receptor binding activity with [^{125}I]CYP. Data are expressed as percent of controls which received drug but no alkylator. N = 2 where the distance between the bars is the range with the data point at the center of it. Data are representative of four experiments.

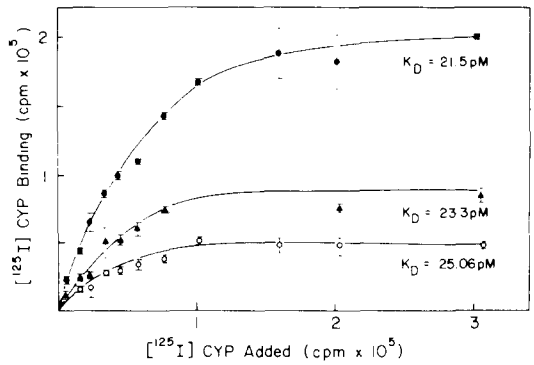

Fig. 4. Saturation binding isotherms of control and alkylated beta-adrenergic receptor. Purified frog red blood cell plasma membranes were reacted with FSNA under conditions to produce an apparent 50% (▲) and 75% (○) loss of binding sites relative to controls (●). Saturation binding isotherms with [^{125}I]CYP were then constructed. Each point was determined in duplicate. Dissociation constants (K_D) were determined by computer analysis. K_d values are shown next to each curve. N = 2 where the distance between the bars is the range with the data point at the center of it. Data are representative of two experiments.

tissues, which contain both beta$_1$- and beta$_2$-adrenergic receptors in varying ratios [11]. Turkey red blood cells contain beta receptors exclusively like the beta$_1$ subtype. As shown in Fig. 2B, inactivation of receptors with FSNA occurred with both beta$_1$ and beta$_2$ particulate receptor preparations obtained from either mammalian lungs or frog and turkey (data not shown) red blood cells. Similar results were obtained with pNBSF. Both agonist (isoproterenol) and antagonist (alprenolol) prevented the loss of radioligand antagonist binding sites when membranes (frog RBC) were pretreated with agonist or antagoinst, and then treated with the alkylating agent. The weakly active dextro isomer of isoproterenol did not prevent inactivation of receptor radioligand binding in frog RBC plasma membranes by FSNA (Fig. 3). Similar results were obtained with plasma membranes prepared from mammalian lungs and turkey red blood cells.

Where inactivation did not proceed to 100% (presumably due to rapid hydrolysis of FSNA and pNBSP in aqueous solution), complete inactivation could be achieved by addition of fresh reagent. That decreases in antagonist binding site number are due to a loss of binding sites rather than a change in receptor affinity for radioligand is shown in Fig. 4 where saturation binding isotherms show a reduction in B_{max} with little change in K_D (inset).

Of the compounds tested, pNBSF has been reported to be specific for tyrosine [12]. FSNA however, might display some reactivity towards cysteine or lysine. To exclude the possibility that loss of receptor antagonist binding sites might be due to alkylation of these residues as well as tyrosine, frog

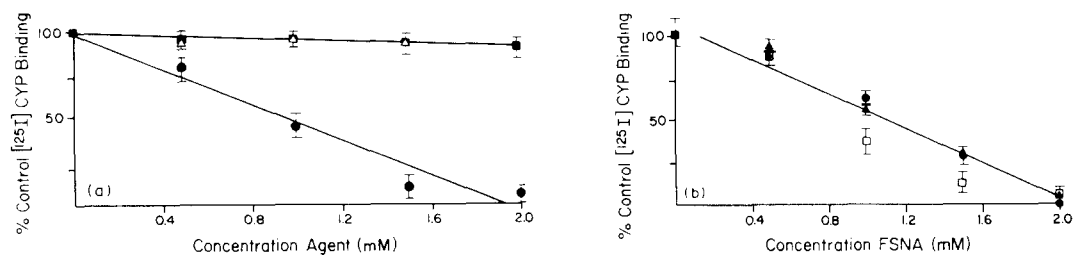

Fig. 5. (a) Interaction of beta receptors with fluorosulfonylnapthoic acid and sulfhydryl-directed reagents. Frog red blood cell plasma membranes were incubated with *n*-ethylmaleimide and iodoacetic acid (△ and ■ respectively), and receptor binding activity was assayed with [^{125}I]CYP. In addition, membranes were preincubated with *n*-ethylmaleimide prior to addition of FSNA and a further 30-min incubation (●). Aliquots of each reaction mixture were assayed in duplicate with [^{125}I]CYP. Data are presented as percent controls which received buffer only. (b) Inactivation of beta receptor by lysine-directed reagents. Purified frog red blood cell plasma membranes were incubated with increasing amounts of Bolton Hunter reagent, in the absence (▲) or presence of agonist (●) or antagonist (□) at 10^{-4} M concentration. Membranes were then washed and assayed in duplicate with [^{125}I]CYP. Data are expressed as percent of controls which received no alkylator. For both panels, N = 2 where the distance between the bars is the range with the data point at the center of it.

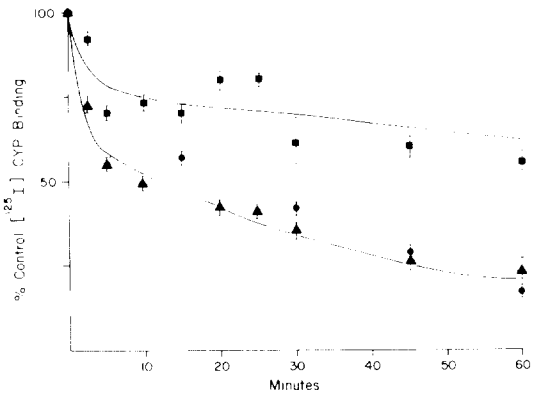

Fig. 6. FSNA inactivation of soluble purified turkey red blood cell beta$_1$-adrenergic receptors. Purified beta$_1$-adrenergic receptors (300 fmoles) obtained from turkey red blood cell plasma membranes [10] were incubated with 10^{-3} M FSNA in the presence of buffer only (●), 10^{-5} M alprenolol (■) or 10^{-5} M isoproterenol (▲). At each time point indicated, aliquots were desalted free of alkylator and drug by Sephadex G-50 chromatography. Receptor binding activity was assayed with [^{125}I]CYP in duplicate and is shown as a percent of control (buffer or drug only) incubations. N = 2 where the distance between the bars is the range with the data point at the center of it. Data are representative of three experiments.

RBC plasma membranes were incubated with *n*-ethylmaleimide and iodoacetic acid. These membranes were chosen since their response to sulfhydryl reagents is well documented. As reported elsewhere [13], in this system these compounds at the concentration used did not inactivate particulate beta-receptor antagonist binding activity. Moreover, preincubation with *n*-ethylmaleimide had no effect on the receptor inactivation induced by FSNA (Fig. 5a). Lysine (or amino) directed reagents, however, such as Bolton Hunter reagent [14] were found to inactivate receptor binding activity. This inactivation could not be prevented by preincubation with adrenergic agents (Fig. 5b).

When soluble purified receptor (turkey RBC: beta$_1$ subtype) was reacted with FSNA or pNBSF and examined in a time–course experiment, inactivation of radioantagonist binding activity was also observed. Antagonists were found to protect against this inactivation, whereas agonists did not (Fig. 6). As was the case for membrane preparations, saturation binding isotherms showed a loss of sites rather than a change in receptor affinity for [^{125}I]CYP. In these time course experiments, inactivation typically proceeded to 70% and, as was observed for particulate membrane preparations, remaining receptor activity could be inactivated by addition of fresh FSNA (data not shown).

DISCUSSION

Although it has been postulated that the agonist binding site of the beta-adrenergic receptor includes an aromatic amino acid [15, 16], direct chemical confirmation has been lacking. In studies presented here, we have demonstrated that two tyrosine directed alkylating reagents inhibit the binding of radiolabeled beta-adrenergic receptor antagonists and that inactivation can be prevented by adrenergic agents. That the inhibition of binding was due to inactivation of receptors rather than alteration in affinity was shown by the generation of saturation binding isotherms and calculation of affinity constants and B_{max} (Fig. 4). Control experiments also demonstrated that the alkylating agents employed did not interact with the radiolabeled antagonist used: excluding this potential explanation for the data. Of the two compounds, pNBSF reacts exclusively with tyrosine [12], while FSNA might weakly interact with cysteine or lysine.

That the inactivation of the beta-adrenergic receptors is due to interactions with tyrosine and not cysteine residues is suggested by several observations. First, previous studies have shown that sulfhydryl residues, while important to receptor function, are probably not present in the agonist/antagonist binding site [12]. Possible interactions with cysteine, however, were explored by examining the effects of cysteine directed compounds both on radiolabeled antagonist binding as well as on the ability of FSNA to inactivate sites. Thus, as reported earlier for the frog red blood cell system, pretreatment of membranes with sulfhydryl reagents did not reduce binding activity [13] nor did preincubation with these agents block FSNA inactivation of receptor. Therefore, the results obtained are unlikely to be due to alkylation of cysteine residues. We have also examined the effect on antagonist binding of lysine or amino directed Bolton Hunter reagent. Although this reagent inactivated receptor, inactivation could not be prevented by incubation with agonist or antagonist. While this does not preclude the presence of lysine at the receptor ligand binding site, it does support the contention that FSNA effects are limited to tyrosine alkylation. As both FSNA and pNBSF showed similar effects in several model systems containing different receptor subtypes, the data also suggest that alkylation of tyrosine residue(s) results in inactivation of antagonist binding in both beta$_1$- and beta$_2$-adrenergic receptor subtypes. This is prevented by preincubation of particulate receptors with either agonist or antagonist ligands. One explanation for this observation is that there is at least one tyrosine at the agonist/antagonist binding site. Alkylation of this tyrosine inactivates the beta-adrenergic receptor, and preincubation with agonists or competitive antagonists prevents this presumably by excluding the alkylator. This would suggest that any accessible tyrosines which might be distal to the agonist/antagonist binding sites are not alkylated or that their alkylation has no effect on the binding activity of the receptor. As the beta-adrenergic receptor has approximately 9 tyrosine residues and 14 lysines [9], this also suggests that the binding site may be less sensitive to alkylation of any tyrosines distal to the binding site than lysines. A second possibility is that both agonist and antagonists induce conformational changes in the receptor that protect against tyrosine alkylation at sites distal to the binding center. This would require agonists and antagonists to induce comparable conformational changes. Furthermore, the conformational changes would be

required not to protect against lysine alkylation. This would, therefore, seem less likely.

In contrast to particulate receptors, purified preparations were protected against inactivation by antagonist but not agonist. Identical results were obtained for both isoproterenol and epinephrine (no protection) and alprenolol and propranolol (protection) eliminating the potential of an effect limited solely to synthetic agonists. One explanation for these observations is that solubilization exposes tyrosine residues normally obscured by the cell membrane and that agonists promote conformational changes which allow a greater extent of tyrosine alkylation by exposing these residues. Alternatively, agonists might enhance the reactivity of a key tyrosine residue(s). Antagonist may protect by site occupancy as proposed for particulate preparations or by induction of conformational changes which make tyrosine(s) exposed on solubilization unavailable. Definitive discrimination between these possibilities will require careful titrations with purified receptor and comparison of alkylated peptides with peptides that bind agonists and antagonists.

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