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Mutational analysis of ligand binding activity of β_2 adrenergic receptor expressed in *Escherichia coli*

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A novel *in situ* screening procedure was used to identify neutral mutations in the human β_2 adrenergic receptor (β_2AR). The coding region of the human β_2AR gene was subcloned under transcriptional control of an inducible T7 promoter and used to transform *Escherichia coli*. Colonies expressing the β_2AR bound the radiolabeled antagonist [^{125}I]iodocyanopindolol and could be identified by autoradiography after transfer to nitrocellulose filters. The region of the β_2AR between residues 76 and 83, in the second transmembrane helix, was mutagenized by a saturation mutagenesis technique, so that virtually all of the β_2AR genes contained at least one mutation. Colonies retaining ligand binding activity were isolated using the *in situ* screen. Sequence analysis of the active mutant receptor genes allowed the identification of individual amino acid side chains which are essential for either ligand binding or structural integrity of the β_2AR receptor.

Key words: β_2 adrenergic receptor/ligand binding/random mutagenesis

Introduction

Eukaryotic plasma membrane receptors mediate two main functions; they bind external ligands and they activate an effector system on the intracellular surface. The adrenergic receptors (α_1, α_2, β_1, β_2, β_3) mediate their physiologic effects *via* G-proteins in response to the binding of the hormones epinephrine and norepinephrine. In addition to these physiologic ligands, they bind a range of synthetic agents that act *in vivo* as agonists or antagonists. Adrenergic receptor subtypes are defined by their characteristic affinities for these ligands. A major focus of molecular pharmacology has been on understanding how this set of structurally homologous receptors display functionally distinct specificities and how structural modification of agonists and antagonists alter their receptor binding properties.

To help answer these questions we have studied receptor–ligand interactions of the human β_2AR using an *Escherichia coli* expression system. This system has several advantages: (i) the human β_2AR is the best characterized of this class of receptors, (ii) β_2AR fusion proteins expressed in *E.coli* have been shown to retain their ligand binding properties (Marullo *et al.*, 1988, 1989, 1990) and (iii) expression of the *E.coli* host allows the facile generation and phenotypic screening of large populations of mutants. In this report we describe a system whereby mutants of β_2AR may be rapidly generated and receptor–ligand interactions assayed. The β_2AR gene is expressed in *E.coli* without fusion to a bacterial protein, and assayed *in situ* for ligand binding activity. Using an oligonucleotide directed random mutagenesis procedure we generated a library of β_2AR variants and screened for receptor activity by detection of bacterial colonies which bound the β_2AR antagonist [^{125}I]iodocyanopindolol (^{125}ICYP). Using this method we screened for receptors bearing neutral mutations in a portion of the second transmembrane domain. Analysis of these mutations allowed us to correlate the variability of the mutagenized positions with ligand binding activity thereby defining residues which are either structurally or functionally important.

Results

To isolate mutants of β_2AR that retain ligand binding activity we developed a system which allows screening for expression of functional human β_2AR in *E.coli*. Bacteria were transformed with plasmid pRB12.65 which contains the coding region of the human β_2AR gene fused to the bacteriophage T7 promoter (Figure 1). Upon induction of the T7 polymerase gene by IPTG, the β_2AR protein is

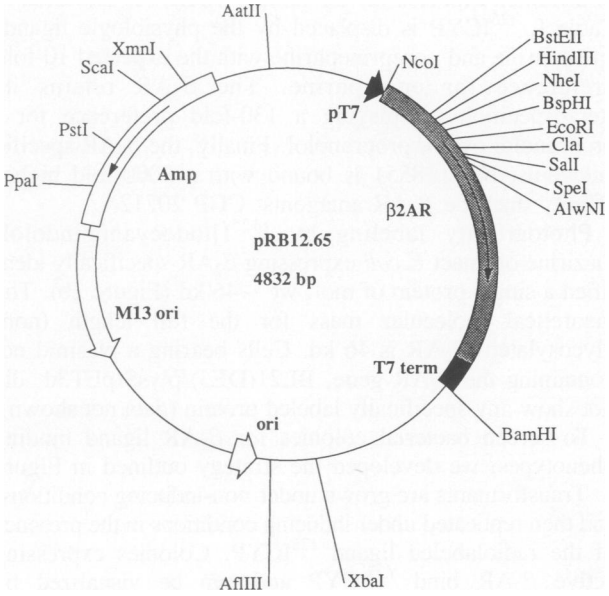

Fig. 1. Structure of the T7/β_2AR expression plasmid. The β_2AR gene is under transcriptional control of the bacteriophage T7 promoter and has the T7 terminator sequences downstream. The coding region of the β_2AR gene in plasmid pRB12.65 has seven new sites in the β_2AR gene: *Hin*dIII, *Nhe*I, *Bsp*HI, *Eco*RI, *Cla*I, *Sal*I and *Spe*I.

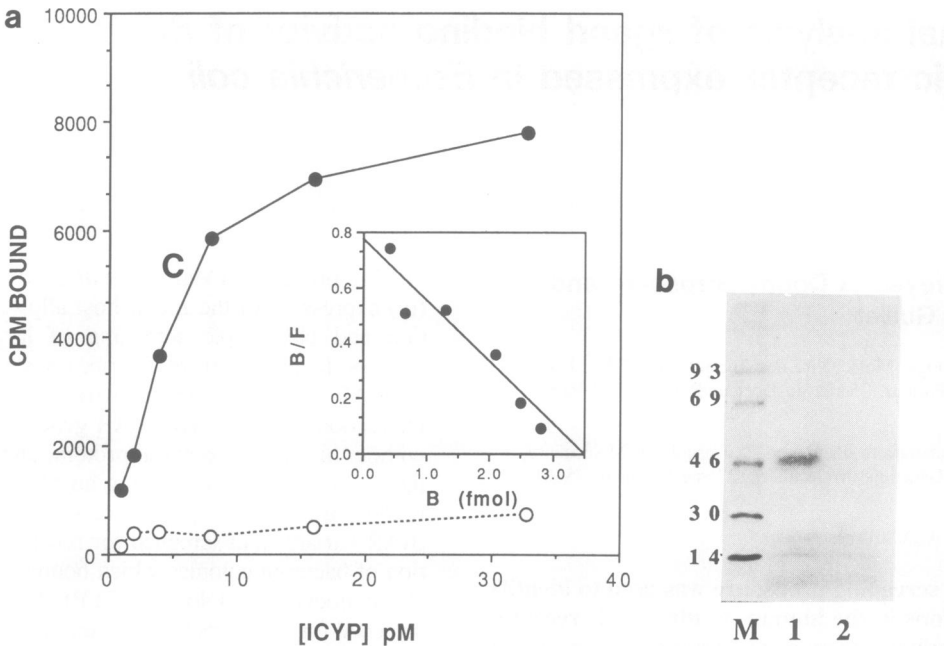

Fig. 2. Expression of β_2AR in *E.coli* strain BL21(DE3)/plysS/pRB12.65. (**a**) (●) Isotherm and Scatchard analysis (inset) of ^{125}ICYP binding to intact *E.coli* expressing the β_2AR. (○) Non-specific binding was determined in the presence of 2 μM *d,l* propranolol. (**b**) Photoaffinity labelling of intact *E.coli* expressing human β_2AR. Cells were labeled with the photoaffinity ligand [^{125}I]iodocyanopindolol-diazirine alone (lane 1) or in the presence of 10 μM *d,l* propranolol (lane 2). Samples were electrophoresed on 9% polyacrylamide–SDS gels. Molecular weight markers (lane M) are indicated on the left. The theoretical molecular mass for the (non-glycosylated) β_2AR is 46 kd.

expressed as determined by ligand binding activity. In liquid culture, binding activity for ^{125}ICYP increased after addition of IPTG and peaked at 6 h (data not shown). As shown in Figure 2(a), the binding of the β_2AR antagonist ^{125}ICYP was saturable, with a calculated K_D of 3.4 pM. The maximum number of sites was ~200 per cell, representing an expression level of ~0.01% of total protein. This binding was displaced by a number of unlabeled ligands and displayed the characteristic stereospecificity and rank order of affinities expected for the human β_2AR. As indicated in Table I, ^{125}ICYP is displaced by the physiologic ligands epinephrine and noreprinephrine with the expected 10-fold preference for epinephrine. The β_2AR retains its stereospecificity displaying a 130-fold preference for *l* propranolol over *d* propranolol. Finally, the β_2AR specific antagonist ICI 118551 is bound with a 3000-fold higher affinity than the β_1AR antagonist CGP 20712A.

Photoaffinity labeling by [^{125}I]iodocyanopindolol-diazirine of intact *E.coli* expressing β_2AR specifically identified a single protein of mol. wt ~46 kd (Figure 2b). The theoretical molecular mass for the full length (non-glycosylated) β_2AR is 46 kd. Cells bearing a plasmid not containing the β_2AR gene, BL21(DE3)/plysS/pET3d, did not show any specifically labeled protein (data not shown).

To screen bacterial colonies for β_2AR ligand binding phenotypes, we developed the strategy outlined in Figure 3. Transformants are grown under non-inducing conditions, and then replicated under inducing conditions in the presence of the radiolabeled ligand ^{125}ICYP. Colonies expressing active β_2AR bind ^{125}ICYP and can be visualized by autoradiography after transfer to nitrocellulose filters. As in the liquid phase and the photoaffinity labeling experiments, the radiolabeled ligand is displaced by the addition of the unlabeled antagonist propranolol (Figure 3).

Table I. Binding of β adrenergic ligands to the human β_2AR expressed in *E.coli*

Competitor	Pharmacological property	K_i (nM)
l propranolol	antagonist	0.13 ± 0.01
d propranolol	antagonist	17.7 ± 1.3
Epinephrine	agonist	725 ± 240
Norepinephrine	agonist	7255 ± 2200
ICI 118551	β_2 antagonist	0.66 ± 0.12
CGP 20712A	β_1 antagonist	2019 ± 500

Competition assays on intact *E.coli* were performed as described in Materials and methods.

Random mutations in the β_2AR gene between the *Hin*dIII and *Nhe*I sites were generated by a cassette method (Oliphant *et al.*, 1986). As the level of mutagenesis was high, most of the receptor genes had multiple mutations, on average 3.3 mutations per gene. The resulting library of mutant receptor genes was introduced into the BL21(DE3)/plysS expression strain by transformation. Using the *in situ* screen described, we screened for receptor genes with neutral mutations, i.e. those which retain ^{125}ICYP binding. Most of the resulting mutant genes were inactive, however, ~25% of the colonies displayed ligand binding. Colonies were purified by restreaking on the same media, retested by a secondary screen of colony patches as described in Materials and methods, and the sequences of their corresponding β_2AR genes determined by DNA sequencing. Figure 4 lists 25 active sequences recovered from the mutagenesis of the *Hin*dIII–*Nhe*I cassette which codes for residues 76–83, lying within the putative second transmembrane domain. These sequences contain as a set, 19 different amino acid substitutions among the eight positions mutagenized. Two

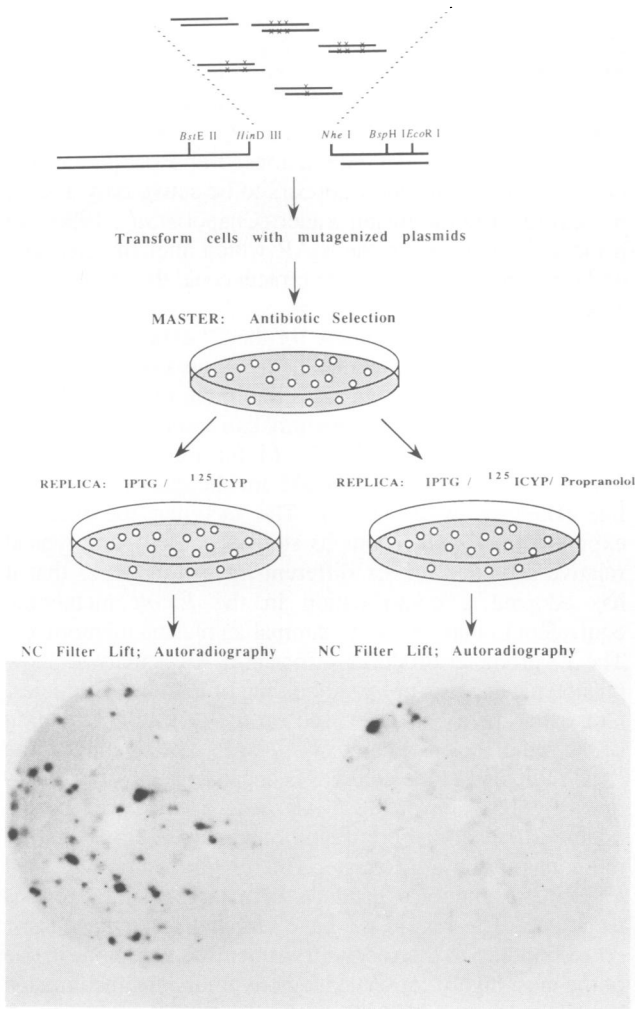

Fig. 3. Mutagenesis/screening strategy. A portion of β_2AR located in the second transmembrane region was mutagenized and candidates screened for ^{125}ICYP ligand binding activity. A population of oligonucleotide cassettes containing an average of 3.3 random changes throughout the cassette were synthesized. Only the first base of codon 83 was mutagenized, as it lies at the end of the cassette. Codon 83 will have both a lower frequency and restricted set of expected mutagenic substitutions (Gly → Arg or Gly → Trp). Cassettes were ligated into the *Hin*dIII–*Nhe*I backbone of plasmid pRB12.65HN, reconstituting the β_2AR gene. *E.coli* were then transformed and screened for ^{125}ICYP binding activity under inducing conditions. Plates contain 20 pM ^{125}ICYP alone, or 20 pM ^{125}ICYP plus 10 μM *d,l* propranolol. Details of the procedures are presented under Materials and methods.

distinct substitution patterns emerge from these experiments: residues which are freely substituted and residues which are conserved. In the sequences comprising the ligand binding set, residues that play essential roles in ^{125}ICYP binding should be conserved, while those that play insignificant roles should be substituted freely. As controls, we sequenced receptor genes from clones which did not bind ligand to confirm that non-conservative mutations had indeed been generated at positions conserved in the set of active receptors (not shown).

Non-conservative substitutions are found at residues Cys77, Val81 and Asp79, suggesting that these side chains are unimportant for ^{125}ICYP binding. Codon 83 was partially mutagenized and we recovered only a single

76	77	78	79	80	81	82	83
ALA	CYS	ALA	ASP	LEU	VAL	MET	GLY
-	SER	-	-	-	PHE	-	-
-	SER	*	GLY	*	LEU	-	-
*	-	-	-	*	-	ARG	-
-	-	-	GLY	-	-	-	-
-	-	-	HIS	-	-	-	-
-	-	-	ASN	-	-	-	-
*	-	-	GLU	-	*	-	-
-	*	-	-	MET	-	-	-
-	*	-	-	-	-	LEU	-
-	-	-	-	-	PHE	-	-
-	PHE	SER	-	-	-	LEU	-
*	TRP	-	HIS	*	-	-	-
VAL	-	*	GLU	-	-	-	-
-	ALA	THR	GLY	-	-	-	-
-	*	-	GLU	-	LEU	-	-
-	GLY	-	*	MET	-	-	-
-	*	-	-	*	-	-	-
*	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-
-	SER	*	HIS	-	PHE	-	-
-	PHE	*	-	-	LEU	-	-
-	*	-	-	-	MET	-	-
-	*	-	-	*	*	-	-
-	SER	-	ASN	-	-	LEU	-
*	PHE	-	-	*	-	-	TRP

Fig. 4. Functional β_2AR sequences. Functional β_2AR sequences following random mutagenesis of the *Hin*dIII–*Nhe*I cassette, corresponding to amino acids 76–83. Dashes indicate that the wild type codon was recovered at that position. Asterisks indicate that a mutant codon which encoded the wild type amino acid was recovered at that position.

substitution, Gly83 → Trp. This non-conservative substitution suggests that position 83 may be able to accommodate a range of amino acids as well. Only conservative changes are found at residues Ala76, Ala78 and Leu80. At Met82 we find an unusual pattern in that this position accepts either the conservative leucine substitution or the positively charged arginine. We include Met82 with the conserved set despite the substitution by an arginine at this position, as the long aliphatic side chain of arginine allows it to substitute for hydrophobic residues under some circumstances (Bowie and Sauer, 1989). The conservative substitution patterns found at positions 76, 78, 80 and 82 indicate that these side chains are important for either antagonist binding or maintenance of receptor structure.

Discussion

The structure of the β_2AR is unknown, but it has been proposed that it inserts into the lipid bilayer in a manner analogous to that of the rhodopsins: a cluster of seven hydrophobic transmembrane α-helices which surround an aqueous ligand binding pocket (Dixon *et al.*, 1986). Ligand interactions with β_2AR have been studied using several approaches: limited proteolysis of receptor protein, deletion analysis of the β_2AR gene, construction of chimeric receptor genes and site directed mutagenesis of the receptor genes. Proteolysis and deletion analysis have identified the putative transmembrane α-helices as the site of receptor–ligand interaction (Dixon *et al.*, 1987a,b; Dohlman *et al.*, 1987a). The question of which individual residues in the protein determine the affinity and specificity of the ligand–receptor interaction has been addressed by the construction of chimeric α/β (Kobilka *et al.*, 1988) and β_1/β_2 receptors (Frielle *et al.*, 1988; Marullo *et al.*, 1990) and by site directed mutagenesis of the cloned hamster (Strader *et al.*, 1987a,b) and human β_2AR genes (Chung

76	77	78	79	80	81	82	83
ALA	CYS	ALA	ASP	LEU	VAL	MET	GLY
VAL	SER	THR	GLY	MET	MET	LEU	TRP
	ALA	SER	HIS		LEU	ARG	
	PHE		ASN		PHE		
	TRP		GLU				
	GLY						

ALA	CYS	ALA	ASP	LEU	VAL	MET	GLY
GLY	SER	THR		MET	MET	LEU	SER
	ALA			ILE	LEU	VAL	ALA
	VAL				ILE	ILE	
	ILE						

Fig. 5. Evolutionarily generated sequences. The set of allowed amino acid substitutions found in the active human β_2AR (**upper panel**) in the mutagenesis experiments, compared with the set of amino acids found at these positions among homologous receptors (**lower panel**): This composite is taken from amino acid sequences of 28 related receptors at their equivalent positions. Sequences used were the human, rat, mouse and hamster β_2AR; human and turkey β_1AR; human β_3AR; human α_2AR; hamster α_1AR; human and rat 5HT receptors; the rat D_2 receptor; human, rat mouse and pig M_1 muscarinic acetylcholine receptors; human, rat and pig M_2 receptors; human, rat and pig M_2 and M_3 receptors; human and rat M_4 and M_5 receptors.

et al., 1988; Fraser *et al.*, 1988; Fraser, 1989). These groups have proposed individual amino acid residues which may be involved in receptor–ligand contacts.

Our efforts have been directed at targeted random mutagenesis experiments that examine the pattern of residue substitutions accommodated at a given position while still permitting function. This approach has been successful in identifying residues involved in protein–protein interactions (Reidhaar-Olson and Sauer, 1988; Breyer and Sauer, 1989), enzymatic activity (Loeb *et al.*, 1989; Oliphant and Struhl, 1989) and in analyzing protein secondary structure (Bowie and Sauer, 1989). We have applied this method for screening functional β_2AR expressed in *E.coli*. The three important features of this system are (i) expression of active receptor without fusion to a bacterial protein, (ii) modification of the β_2AR gene to include convenient restriction sites to facilitate mutagenesis, and (iii) an *in situ* screening procedure for the detection of ligand binding activity in colonies, allowing the screening of a large number of mutants without the need for a liquid phase ligand binding assay.

It has been previously shown that the human β_2AR retains its ligand binding properties when it is expressed in *E.coli* as a fusion with *βgal* (Marullo *et al.*, 1988), the outer membrane protein *lamB* (Marullo *et al.*, 1989) or the periplasmic protein *malE* (J.-G.Guillet, unpublished observations). Here we show that the β_2AR can in fact be expressed without fusion in its active form. The maximum expression level for any of the above mentioned bacterial expression systems is ~250 binding sites per cell. The low level of receptor expression appears to be independent of the promoter (*tac, malE* or T7) and may be due to an intrinsic capacity of the *E.coli* membrane for the receptor. Nevertheless, the expression level of β_2AR in *E.coli* is ~10 fold higher than that found in the human A431 cell line, taken as a percentage of total protein.

Expression of the native β_2AR protein allows study of a homogeneous receptor population rather than the mixture of full length fusion protein and degradation products obtained in the *lamB* and *βgal* expression systems. While previous studies with β_2AR fusion proteins suggest that the protein fusion does not interfere with receptor function, mutations of the β_2AR could result in novel receptor–fusion leader interactions. For example, in the *lamB* system the receptor appears to be active only after it is cleaved from the fusion leader (Chapot *et al.*, 1990). In principle, mutations in the β_2AR which alter the cleavage of the receptor from the fusion protein could therefore inhibit receptor function.

The K_D value reported here for the T7 expression system is close to the values obtained in mammalian expression systems. There is a 7-fold variation in the reported affinity of β_2AR for ^{125}ICYP in mammalian expression systems with values ranging from 21 pM for transfected L-cells (Chung *et al.*, 1988) to 134 pM for the human A431 cell line (Bouvier *et al.*, 1987). The fact that the receptor expressed in *E.coli* retains its stereospecificity and typical relative affinities for six different ligands indicates that it has adopted a conformation in the *E.coli* membrane equivalent to that found in mammalian plasma membranes. The T7 promoter produces sufficient β_2AR activity to allow phenotypic analysis of ligand binding of individual colonies. Our experiments have focused on the analysis of binding of the radiolabeled antagonist ^{125}ICYP. The binding of the ^{125}ICYP ligand to the colonies is dependent upon the induction of expression of the β_2AR gene, is displaced by the unlabeled antagonist propranolol and can be eliminated by mutations in the β_2AR gene. This *in situ* screen was used to identify functional products of a targeted mutagenesis technique. The region we have targeted for mutagenesis, corresponding to the second transmembrane region, is one of the most highly conserved regions among the mammalian hormone receptors (Dohlman *et al.*, 1987b).

Residues conserved in the mutagenesis experiments, Ala76, Ala78, Leu80 and Met82, are presumably required for ^{125}ICYP binding directly or for maintaining the folded receptor structure. In the latter case these positions might define contact residues in helix–helix packing interactions. Of the variable positions, 77 and 81 accept a number of hydrophobic substitutions. In an α-helical conformation, positions 77 and 81 would lie on the same face of the helix separated by one turn. The variability and hydrophobicity of these positions fit the criteria for residues which define the membrane exposed side of a transmembrane helix (Rees *et al.*, 1989). The two remaining non-conserved positions, 79 and 83, are located on one face of the helix, displaced by half a helical repeat from positions 77 and 81. Asp79 can be replaced by Gly, His, Asn and Glu, a substitution pattern which supports models of β_2AR where Asp79 lies in an aqueous cavity formed by the seven transmembrane helices.

Figure 5 compares the substitution pattern of 28 homologous hormone receptors with the substitution pattern found in the random mutagenesis experiments. Residues which are evolutionarily conserved may be critical for maintenance of the overall structure, hormone binding, G-protein coupling, desensitization or interaction with regulatory molecules such as βARK or βArrestin. The mutagenesis technique combined with a screen for antagonist binding relaxes the requirements imposed on receptor structure, as it demands neither signal transduction nor effector

coupling. In general the variability between the two sets shows a good correlation with respect to number and types of substitutions allowed at each position. For example, in the mutagenesis experiments we recovered methionine in addition to the wild type leucine at position 80 and among the naturally occurring variants only leucine, methionine and isoleucine are found at the equivalent position. Similarly at position 78 we recover serine and threonine as well as the wild type alanine whereas among the 28 homologous receptors only alanine and threonine are found. An exception to this correlation is seen at Asp79 which is strictly conserved among the set of naturally occurring receptors, suggesting that it plays a critical role for either structure or function. In contrast, in the mutagenesis experiments we found four different amino acid substitutions including histidine and glycine, suggesting that Asp79 is required neither for proper folding of the receptor in the membrane nor for adopting the antagonist binding conformation and could be considered a residue of 'low informational content' (Reidhaar-Olson and Sauer, 1988). Taken together these findings indicate that Asp79 plays a crucial functional role e.g. in signal transduction by bound hormone, but not a role critical for structure or antagonist binding.

We have concentrated on a small, highly conserved region of β_2AR, studying effects of mutations on antagonist binding. This technique can be applied throughout the receptor gene, employing a number of variations on the screen. For example, in an analogous fashion to the liquid phase competition experiments of the type presented in Table I, it should in principle be possible to include unlabeled agonist as a competitor in the assay plates. By including epinephrine as well as ^{125}ICYP in the plates, only variants which bind ^{125}ICYP but *not* epinephrine will be isolated. In this way residues important for agonist and antagonist binding may be distinguished. Using chimeric receptors, Marullo *et al.* (1990), have shown that there is no single region of β adrenergic receptors which defines subtype selectivity. By using the screening procedure described here it should be possible to identify positions within the receptor genes critical for defining subtype selectivity. We expect that this system may be adapted to any of the cloned receptors for which labeled ligands are available.

Materials and methods

Strains and plasmids

Plasmid pRB12.65 is a derivative of the M13 origin plasmid pZ150 (Zagursky and Berman, 1984). It encodes ampicillin resistance and the human β_2AR (Figure 1). The β_2AR gene is under transcriptional control of the bacteriophage T7ϕ10 promoter and has T7 terminator sequences downstream (Studier and Moffat, 1986). These T7 sequences are derived from the expression vector pET3d, a gift from W.Studier. The *Nco*I–*Bam*HI fragment containing the β_2AR coding region derived from plasmid 973 (Marullo *et al.*, 1989) replaces the small *Nco*I–*Bam*HI fragment between the T7 promoter and terminator sequences of pET3d. The resulting T7/β_2AR fusion uses the ATG at codon one of the β_2AR gene for translation initiation. The coding region of the β_2AR gene in plasmid pRB12.65 has been modified to contain several additional restriction sites not present in the wild type gene (Emorine *et al.*, 1987; Kobilka *et al.*, 1987). These sites were introduced by replacing the 258 bp *Bst*EII–*Alw*NI restriction fragment of the β_2AR gene with a synthetic fragment containing 17 silent mutations. These mutations result in the introduction of seven new restriction sites in the β_2AR gene: *Hin*dIII at codons 73/74/75, *Nhe*I at codons 83/84/85, *Bsp*HI at codons 95/96/97, *Eco*RI at codons 107/108, *Cla*I at codons 119/120/121, *Sal*I at codons 129/130 and *Spe*I at codons 136/137. Plasmid pRB12.65HN is identical to plasmid pRB12.65 except that the region encoding residues 73–85 (the small *Hin*dIII–*Nhe*I fragment) has been replaced with a 0.7 kb 'stuffer' fragment.

Plasmid plysS is a derivative of pACYC and encodes chloramphenicol resistance and T7 lysozyme (Studier *et al.*, 1990).

Strain BL21(DE3) (F^- *hsdS gal* r^- m^-) contains the prophage DE3 bearing the T7 polymerase gene under transcriptional control of the inducible plac UV5 promoter (Studier and Moffat, 1986). Strain X90 is *argE* Δ*lac pro*/F' lac I^Q *lac*$^+$ *pro*$^+$ (Amman *et al.*, 1983).

Oligonucleotide cassette mutagenesis

Oligonucleotide cassettes with high levels of random mutations were prepared by the self priming method of Oliphant *et al.* (1986). One strand of the cassette was synthesized chemically with 77% of the wild type base and 7.7% of the other three bases included at each mutagenized position. The complementary strand was synthesized enzymatically, double stranded cassettes prepared, and the resulting mutagenic cassettes ligated into the *Hin*dIII–*Nhe*I backbone fragment of plasmid pRB12.65HN reconstituting the β_2AR gene.

DNA sequence analysis

For DNA sequence analysis, plasmid DNA was purified from *E.coli* strain BL21(DE3)/plysS/pRB12.65 by the alkaline lysis method (Maniatis *et al.*, 1982). Plasmid DNA was transformed into strain X90 and single stranded plasmid DNA was purified from an M13 R408 transducing lysate. DNA sequences were determined by the dideoxy method (Sanger *et al.*, 1977) using the T7 Sequenase enzyme (United States Biochemical Corp.).

Induction in liquid culture

BL21(DE3)/plysS/pRB12.65 was grown in M9 media (Miller, 1972) containing 0.4% glucose, 100 μg/ml ampicillin and 25 μg/ml chloramphenicol at 37°C. At an A_{600} between 0.2 and 0.3, IPTG was added to a final concentration of 0.5 mM and the cultures incubated a further 6 h at 23°C.

Binding assays

Ligand binding assays on intact *E.coli* were essentially as described (Marullo *et al.*, 1988). Saturation experiments were performed with 1×10^7 cells in a volume of 1 ml, and varying concentrations of ^{125}ICYP (Amersham) for 1 h at 37°C. Non-specific binding was determined in the presence of 2 μM *d,l* propranolol. Competition assays were performed using 2×10^7 cells, 8 pM ^{125}ICYP and various concentrations of unlabeled competitor.

Photoaffinity labeling

Intact *E.coli* were photoaffinity labeled with [^{125}I]iodocyanopindolol diazirine (Amersham) as described (Marullo *et al.*, 1989). Non-specific labeling was determined in the presence of 10 μM *d,l* propranolol. Labeled bacteria were solubilized in sample buffer containing 10% SDS for 1 h at 0°C and electrophoresed on 9% polyacrylamide–SDS gels (Laemmli, 1970).

In situ ICYP screening protocol

Mutagenized plasmids were transformed into *E.coli* strain BL21(DE3)/plysS and transformants resistant to ampicillin and chloramphenicol were selected by overnight growth at 37°C, on LBA100CAM25 plates (LB agar plates containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol).

To screen for ligand binding, master transformation plates were replicated onto plates prepared by overlaying 22 ml LBA100CAM25 with 3 ml top agarose containing ^{125}ICYP to a final concentration (in 25 ml) of 20 pM and IPTG to a final concentration of 0.5 mM. In some cases unlabeled *d,l* propranolol was added to the plates as a competitor at a final concentration of 10 μM. Replica plates were incubated for 15 h at 37°C. Colonies were then lifted from the replica plates onto nitrocellulose filter circles, and exposed to X-ray film for 4–18 h.

In some experiments transformants were plated on M9A100CAM25 plates (M9 agar plates containing 0.4% glucose, 100 μg/ml ampicillin and 25 μg/ml chloramphenicol) and incubated for 36 h. Where master plates were made in M9, all subsequent selections were made on M9A100CAM25 plates, and top agarose was M9 1.5% agarose.

Colonies displaying either positive or negative ^{125}ICYP binding phenotypes were located on the master plate using the ^{125}ICYP probed replica filters as guides. Representative candidates of each phenotype were picked and restreaked for single colonies on appropriate antibiotic selection plates. To confirm the ^{125}ICYP binding phenotypes, colonies were patched onto master plates. Replicas were made on LB or M9 A100CAM25 plates containing 20 pM ^{125}ICYP and 0.5 mM IPTG, reincubated for 6 h, and filter lifts made as above.

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