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Preliminary communication

Site-directed mutagenesis of the rat β_1-adrenoceptor. Involvement of Tyr$^{356\ (7.43)}$ in (+/–)cyanopindolol but not (+/–)[^{125}Iodo]cyanopindolol binding

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Abstract

To determine the role played by Tyr$^{356\ (7.43)}$ in the rat β_1-adrenoceptor in binding the antagonists (+/–)cyanopindolol (4-[3-(*t*-butylamino]-3-(2′-cyano-indoloxy)-2-propanolol) and its iodinated analogue (+/–)[^{125}Iodo]cyanopindolol (1-(*t*-butylamino]-3-(2′-cyano-3′-iodo-indoloxy)-2-propanolol), Tyr$^{356\ (7.43)}$ was mutated to either Phe or Ala and binding affinities determined for wild type and mutant rat β_1-adrenoceptors. Our results indicate that Tyr$^{356\ (7.43)}$ is important for (+/–)cyanopindolol, but not (+/–)[^{125}Iodo]cyanopindolol, binding and that (+/–)cyanopindolol adopts a "reverse" binding orientation whereas (+/–)[^{125}Iodo]cyanopindolol cannot be accommodated in this binding mode. We define a "reverse" antagonist binding mode as one where the aryloxy moiety interacts with residues on transmembrane helices 1, 2, 3 and 7. The β_1-adrenoceptor site-directed mutagenesis results are the first to support a "reverse" antagonist binding orientation and the involvement of Tyr$^{356\ (7.43)}$ in this binding mode.

Keywords: β_1-Adrenoceptor; Molecular modelling; (+/–)cyanopindolol; (+/–)[^{125}Iodo]cyanopindolol; Mutagenesis

1. Introduction

There are few published mutagenesis studies examining the requirements for antagonist binding to the β-adrenoceptor and most relate to the β_2-adrenoceptor [1–5]. Asp$^{3.32}$ plays an essential role in the β_2-adrenoceptor by interacting with the amino nitrogen of the antagonist [3] and substitution of Asn$^{7.39}$ by Ala reduces the affinity of aryloxypropanolamines such as propranolol and alprenolol, perhaps by preventing the formation of a hydrogen bond between Asn$^{7.39}$ and the aryloxy oxygen atom of these antagonists [1].

The present study utilised the rat β_1-adrenoceptor to examine the binding orientation and points of interaction for two structurally similar antagonists, i.e. (+/–)-4-[3-(*t*-butylamino]-3-(2′-cyano-indoloxy)-2-propanolol ((+/–)cyanopindolol) and its iodinated form (+/–)-1-(*t*-butylamino]-3-(2′-cyano-3′-iodo-indoloxy)-2-propanolol ((+/–)[^{125}Iodo]cyanopindolol) (Fig. 1A,B). (+/–)[^{125}Iodo]cyanopindolol is of particular interest as its low dissociation constant, in combination with its high specific radioactivity (2175 Ci mmole) and its specificity for β-adrenoceptors has made it the most frequently used radioligand for β_1- and β_2-adrenoceptors [6]. In order to visualise (+/–)[^{125}Iodo]cyanopindolol and (+/–)cyanopindolol binding a model of the seven transmembrane (tm) regions of the rat β_1-adrenoceptor, based on the recently determined three-dimensional crystal structure of bovine rhodopsin [7], was constructed and used in antagonist/receptor docking studies. When manually docking the antagonists into the receptor model it was assumed that interactions between (i) Asp$^{138\ (3.32)}$ and the antagonist amino

Abbreviations: (+/–)cyanopindolol, (+/–)-4-[3-(*t*-butylamino]-3-(2′-cyano-indoloxy)-2-propanolol; (+/–)[^{125}Iodo]cyanopindolol, (+/–)-1-(*t*-butylamino]-3-(2′-cyano-3′-iodo-indoloxy)-2-propanolol; CHO, chinese hamster ovary cells; tm, transmembrane; SEM, standard error of the mean.

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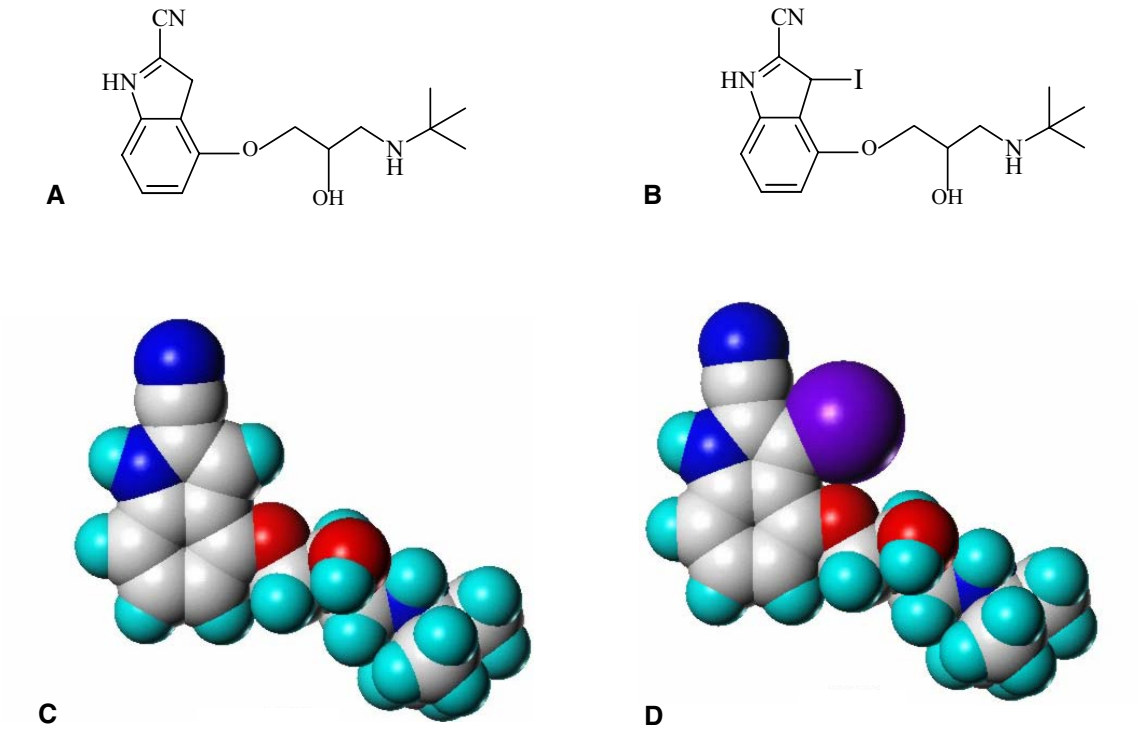

Fig. 1. Chemical structures of **A** (+/–)cyanopindolol and **B** (+/–)[^{125}Iodo]cyanopindolol. The three-dimensional space filling models of **C** (*S*)-cyanopindolol and **D** (*S*)-[^{125}Iodo]cyanopindolol in a low energy extended conformation. The colour coding of the compounds is consistent with the traditional nomenclature: oxygen, *red*; nitrogen, *dark blue*; carbon, *white*; hydrogen, *cyan* and iodine, *purple*.

nitrogen and (ii) Asn$^{352\ (7.39)}$ and the antagonist were essential for antagonist binding. We observed during the antagonist docking studies that if Asn$^{352\ (7.39)}$ interacted with the aryloxy oxygen atom of the antagonist (as suggested by Suryanarayana and Kobilka [1]) then the aryl moiety was located in a hydrophobic pocket defined by residues in tms 1, 2, 3 and 7. Our modelling studies further suggested that one of the aromatic residues in this hydrophobic pocket, Tyr$^{356\ (7.43)}$, was ideally situated to directly interact with the antagonist aryl moiety. This antagonist binding mode resembles one previously described by Lewell [2] and we define it here as the "reverse" antagonist binding mode since it contrasts with the more classical ideas of antagonist binding, i.e. where the antagonist aryloxy ring interacts with a hydrophobic binding pocket involving residues in tms 5 and 6, while the antagonist maintains interactions with Asp$^{138\ (3.32)}$ and Asn$^{352\ (7.39)}$ [5,8–10].

In the present study we mutated Tyr$^{356\ (7.43)}$ to either Phe (retaining an aromatic interaction) or Ala (removing an aromatic interaction) and analysed the effects on (+/–)[^{125}Iodo]cyanopindolol and (+/–)cyanopindolol binding to the rat β_1-adrenoceptor.

2. Results

2.1. Radioligand binding studies

In Table 1 the affinities of (+/–)cyanopindolol and its iodinated derivative, (+/–)[^{125}Iodo]cyanopindolol for the wild type and mutant rat β_1-adrenoceptors are summarised. Saturation binding experiments yielded similar K_d and B_{max} values, although (+/–)[^{125}Iodo]cyanopindolol was slightly less potent (three fold) at Y7.43A mutant receptors compared to wild type. The affinity of (+/–)cyanopindolol for Y7.43A mutant receptors differed greatly (5680-fold) with respect to wild type, while retaining the aromatic interaction in the Y7.43F mutation maintained the binding affinity at wild type levels.

2.2. Antagonist interactions with the rat β_1-adrenoceptor

The (+/–)cyanopindolol binding results for the wild type and mutant rat β_1-adrenoceptors suggests that there is a direct aromatic interaction with Tyr$^{356\ (7.43)}$ (Table 1). When (*S*)-

Table 1
Affinity values (nM) for (+/–)cyanopindolol and (+/–)[^{125}Iodo]cyanopindolol binding in membranes from CHO cells expressing wild type or mutant rat β_1-adrenoceptors

	Affinity		B_{max} (fmol/mg)
	(+/–)Cyanopindolol (K_i) [a]	(+/–)[^{125}Iodo]cyanopindolol (K_d) [b]	
Receptor			
Wild type	0.05 ± 0.002	0.1 ± 0.002	417 ± 132
Y7.43A	284 ± 27 (5680) *,[c]	0.3 ± 0.02 (3) **,[c]	689 ± 91
Y7.43F	0.05 ± 0.0003	0.1 ± 0.01	256 ± 30

Data are mean ± SEM of 3–5 experiments; * $P < 0.001$ and ** $P < 0.01$.
[a,b] were obtained as described under 'Section 5'. All curves were monophasic (Hill coefficient $n_H = 0.89–1$).
[c] Values in parentheses represents the fold changes in affinity relative to wild type.

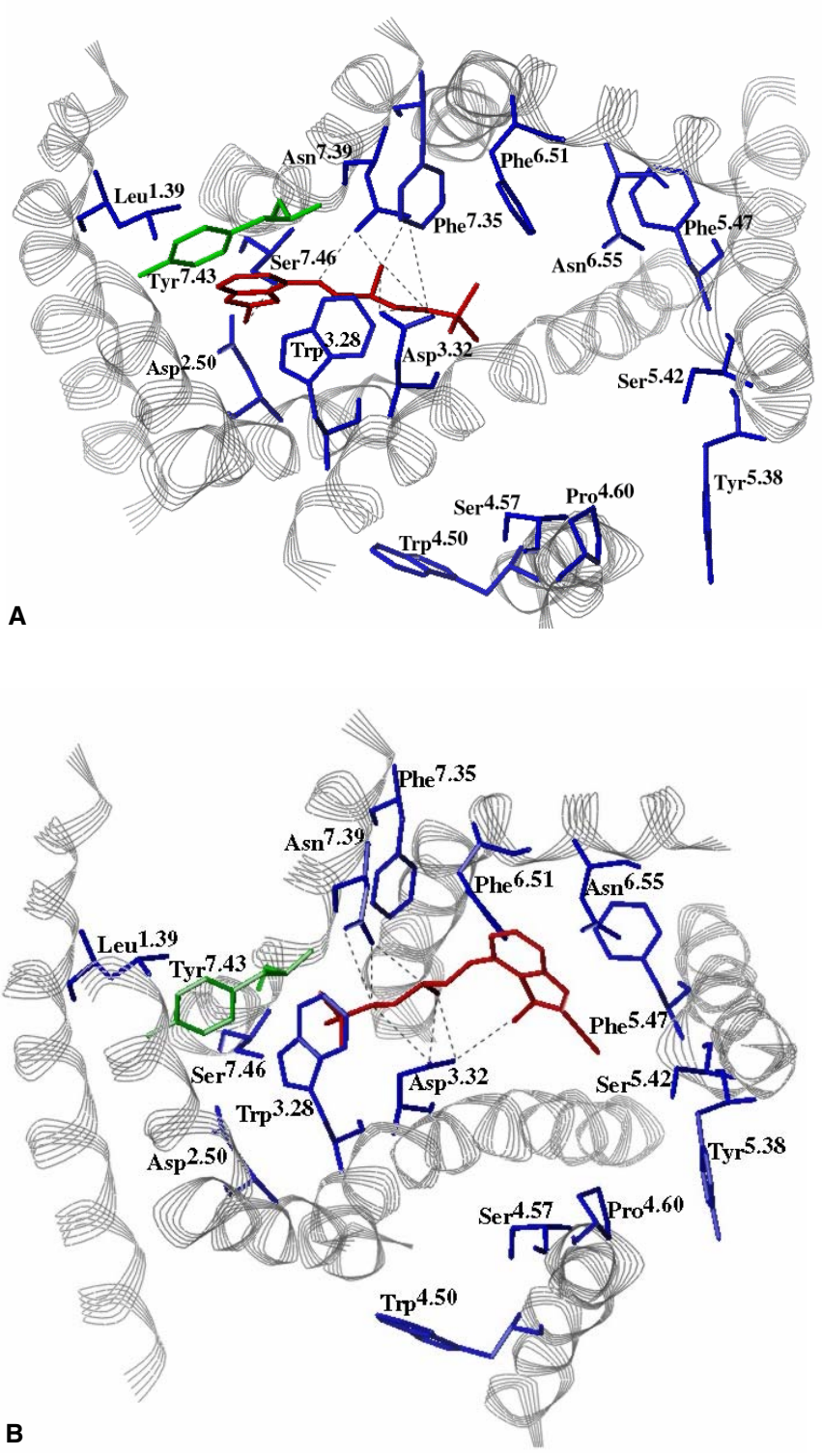

Fig. 2. A model of the rat β_1-adrenoceptor showing **A** (*S*)-cyanopindolol binding in the "reverse" mode and **B** one of the proposed binding orientations of (*S*)-[^{125}Iodo]cyanopindolol. **A** and **B** depict extracellular views looking down through the ligand binding site. The antagonists are coloured red. The tm regions are shown as grey ribbons, the loops were not modelled. Tyr$^{356\ (7.43)}$ mutated in this study is shown in green. Residues in blue are some of the residues discussed in the text. Putative hydrogen bonds are shown as dashed lines, for clarity not all of the interactions are shown. The views of **A** and **B** are not identical and have been adjusted to allow visualisation of the key interactions between the two antagonists and the receptor (see text).

cyanopindolol is docked into the three-dimensional model of the wild type rat β_1-adrenoceptor and interactions with Tyr$^{356\ (7.43)}$, Asp$^{138\ (3.32)}$ and Asn$^{352\ (7.39)}$ are maintained, (*S*)-cyanopindolol adopts the "reverse" antagonist binding mode (Fig. 2A). In this binding orientation, the aryloxy moiety of (*S*)-cyanopindolol sits in a hydrophobic pocket defined by Leu$^{65\ (1.39)}$, Met$^{107\ (2.53)}$, Val$^{111\ (2.57)}$, Val$^{112\ (2.58)}$, Gly$^{115\ (2.61)}$, Trp$^{134\ (3.28)}$, Asn$^{352\ (7.39)}$, Trp$^{353\ (7.40)}$ and

Tyr$^{356\ (7.43)}$. Both Tyr$^{356\ (7.43)}$ and Trp$^{353\ (7.40)}$ make edge-to-face π–π interactions with the aryl ring of (*S*)-cyanopindolol. The cyano group can hydrogen bond to Ser$^{359\ (7.46)}$. Asn$^{352\ (7.39)}$ and Asp$^{138\ (3.32)}$ can interact with both the amine nitrogen and the β-hydroxyl group of (*S*)-cyanopindolol. Asn$^{352\ (7.39)}$ is also able to hydrogen bond to the (*S*)-cyanopindolol aryloxy oxygen. The *t*-butyl group is surrounded by Trp$^{134(3.28)}$, Phe$^{348\ (7.35)}$, Phe$^{329\ (6.51)}$, Val$^{139\ (3.33)}$ and Thr$^{135\ (3.29)}$. (*S*)-Cyanopindolol cannot interact directly with Tyr$^{356\ (7.43)}$ when the antagonist is docked in the "classical" antagonist binding mode, i.e. where the aryloxy moiety is orientated towards tms 5 and 6 and interactions with Asp$^{138\ (3.32)}$ and Asn$^{352\ (7.39)}$ are maintained; nor can it interact with Tyr$^{356\ (7.43)}$ when binding in variations of the "classical" antagonist mode.

In contrast, the (+/–)[^{125}Iodo]cyanopindolol binding results suggest that there is no direct aromatic interaction between Tyr$^{356\ (7.43)}$ and (+/–)[^{125}Iodo]cyanopindolol (Table 1). When attempting to dock (*S*)-[^{125}Iodo]cyanopindolol into the three-dimensional model of the wild type rat β_1-adrenoceptor in the "reverse" antagonist binding mode the (*S*)-[^{125}Iodo]cyanopindolol conformation was energetically unstable (the ΔH_f^0 for the conformer was calculated using the AM1 Hamiltonian within the MOPAC program, the conformer geometry was found not to be a stationary point) and therefore this does not appear to be a feasible binding mode for (*S*)-[^{125}Iodo]cyanopindolol. (*S*)-[^{125}Iodo]cyanopindolol can however be easily accommodated in the rat β_1-adrenoceptor ligand binding site in several other binding modes. In the "classical" antagonist binding mode, the aryl ring of (*S*)-[^{125}Iodo]cyanopindolol is positioned near tms 5 and 6, interacting with Phe$^{233\ (5.47)}$, Phe$^{329\ (6.51)}$ and Phe$^{330\ (6.52)}$ via edge-to-face π–π interactions. The cyano group can hydrogen bond with Ser$^{232\ (5.46)}$ and also participates in π–π interactions with Phe$^{233\ (5.47)}$. The iodine sits in the receptor channel surrounded by Asp$^{138\ (3.32)}$, Val$^{139\ (3.33)}$, Val$^{142\ (3.36)}$ and Trp$^{326\ (6.48)}$. The aryloxy oxygen can hydrogen bond to Asn$^{333\ (6.55)}$. Both the antagonist β-hydroxyl and amine nitrogen can interact with Asp$^{138\ (3.32)}$. The *t*-butyl is surrounded by Trp$^{134\ (3.28)}$, Thr$^{135\ (3.29)}$, Asp$^{138\ (3.32)}$, Phe$^{329\ (6.51)}$, Phe$^{348\ (7.35)}$ and Asn$^{352\ (7.39)}$. However, in the "classical" antagonist binding mode a direct interaction between Asn$^{352\ (7.39)}$ and the (*S*)-[^{125}Iodo]cyanopindolol amine nitrogen is not possible due to the *t*-butyl group. The same is true when (*S*)-[^{125}Iodo]cyanopindolol is docked in a binding orientation consistent with that described for [^{125}Iodo]aminoflisopolol in the β_2-adrenoceptor [11]. In this binding orientation the aryloxy ring system of (*S*)-[^{125}Iodo]cyanopindolol is located near tms 4 and 5 and interacts with Tyr$^{224\ (5.38)}$ (via edge-to-face π–π interactions). Both the iodine and cyano interact with Ser$^{228\ (5.42)}$, while the amine nitrogen and β-hydroxyl both interact with Asp$^{138\ (3.32)}$. The (*S*)-[^{125}Iodo]cyanopindolol *t*-butyl group is surrounded by Trp$^{134\ (3.28)}$, Thr$^{135\ (3.29)}$, Asp$^{138\ (3.32)}$, Trp$^{326\ (6.48)}$, Phe$^{329\ (6.51)}$, Phe$^{348\ (7.35)}$ and Asn$^{352\ (7.39)}$. As is the case for the "classical" binding mode, Asn$^{352\ (7.39)}$ is unable to interact directly with (*S*)-[^{125}Iodo]cyanopindolol when it binds in this orientation. When (*S*)-[^{125}Iodo]cyanopindolol interacts with the rat β_1-adrenoceptor in a variation of the "classical" antagonist binding mode, i.e. the aryl ring is located near tms 6 and 7 (Fig. 2B.), both Asn$^{352\ (7.39)}$ and Asp$^{138\ (3.32)}$ are able to interact with the antagonist amine nitrogen and the β-hydroxyl. The iodine is ideally located to form a further interaction with Asp$^{138\ (3.32)}$. The aryl ring forms edge-to-face π–π interactions with Phe$^{348\ (7.35)}$ and Phe$^{329\ (6.51)}$. Asn$^{333\ (6.55)}$ also interacts with the (*S*)-[^{125}Iodo]cyanopindolol aryl moiety. Phe$^{233\ (5.47)}$, which forms part of the hydrophobic binding pocket for antagonists binding in the "classical" orientation, does not interact with the aryl ring of (*S*)-[^{125}Iodo]cyanopindolol as the distance between the two aryl ring systems is >12 Å. The (*S*)-[^{125}Iodo]cyanopindolol cyano group points towards Tyr$^{224\ (5.38)}$ but does not make any direct interaction with this residue. The *t*-butyl group of (*S*)-[^{125}Iodo]cyanopindolol is surrounded by Val$^{111\ (2.57)}$, Trp$^{134\ (3.28)}$, Thr$^{135\ (3.29)}$, Asp$^{138\ (3.32)}$, Asn$^{352\ (7.39)}$ and Trp$^{353\ (7.40)}$.

3. Discussion

More than one feasible binding orientation may exist for antagonists, as they are not required to induce receptor activation and hence may have fewer binding restrictions compared to agonists. Our site-directed mutagenesis studies, in conjunction with molecular modelling, identified marked differences in the manner in which the structurally similar antagonists (+/–)cyanopindolol and (+/–)[^{125}Iodo]cyanopindolol interact with the β_1-adrenoceptor.

The "reverse" antagonist binding mode proposed on the basis of the mutagenesis studies for (+/–)cyanopindolol, i.e. the aryloxy moiety is positioned towards tms 1, 2, 3 and 7 (Fig. 2A), supports the binding mode previously suggested for some antagonists binding to β_2-adrenoceptors [1,2]. The introduction of iodine into the (+/–)cyanopindolol structure to produce (+/–)[^{125}Iodo]cyanopindolol severely restricts the conformational flexibility of the molecule, increases its physical size (Fig. 1C,D) and prevents the aryloxy moiety of (+/–)[^{125}Iodo]cyanopindolol from binding towards tms 1, 2, 3 and 7. Hence, (+/–)[^{125}Iodo]cyanopindolol cannot adopt the "reverse" antagonist binding mode, however it can be accommodated in the "classical" antagonist binding mode or a variation of this (one example is given in Fig. 2B). The molecular modelling results therefore indicate that the physical features of the antagonist will have a direct bearing on the way in which the compound will bind into the receptor channel. This finding is supported by the binding data (Table 1), as the Y7.43A mutant did not display a large reduction in affinity compared to wild type receptor, which would be expected if an aromatic interaction between Tyr$^{356\ (7.43)}$ and (+/–)[^{125}Iodo]cyanopindolol was lost. The observed reduction in affinity (three fold) for (+/–)[^{125}Iodo]cyanopindolol by the Y7.43A mutant compared to wild

type receptor is minimal and may reflect the result of the mutation on the alignment or stabilisation of the residues that directly interact with the antagonist. In comparison, the 5680-fold reduction in affinity for the Y7.43A mutant observed for (+/–)cyanopindolol is restored to wild type levels by the Y7.43F mutation, providing strong evidence for a direct aromatic interaction between the aryloxy moiety of (+/–)cyanopindolol and $Tyr^{356\ (7.43)}$. The only way a direct aromatic interaction could occur between the aryloxy moiety of (+/–)cyanopindolol and $Tyr^{356\ (7.43)}$ is when the "reverse" antagonist binding mode is adopted.

4. Conclusion

Our work has attempted to explain differences in the way (+/–)cyanopindolol and its iodinated form, (+/–)[^{125}Iodo]cyanopindolol interact with the β_1-adrenoceptor. The combination of molecular modelling and site-directed mutagenesis studies identified a Tyr residue on tm7 as critical for (+/–) cyanopindolol but not (+/–)[^{125}Iodo]cyanopindolol binding, thus highlighting differences in the manner in which these two structurally similar compounds interact with the β_1-adrenoceptor. The site-directed mutagenesis results are of particular interest as they provide the first direct evidence for antagonist binding in a "reverse" mode, i.e. with the aryloxy moiety positioned towards tms 1, 2, 3 and 7, and the involvement of $Tyr^{356\ (7.43)}$ in this binding mode.

5. Experimental protocols

5.1. Materials

Oligonucleotides, geneticin (G418) and the mammalian expression vector pcDNA3.1 were obtained from Invitrogen (Groningen,The Netherlands). Chinese hamster ovary (CHO) cell line, foetal calf serum and bovine serum albumin (BSA) (fraction V) were obtained from Commonwealth Serum Laboratories (CSL) (Parkville, Vic., Australia). Hams F12, sodium pyruvate, trypsin, penicillin and streptomycin were obtained from Edward Keller (Hallam, Vic, Australia). Restriction enzymes *Eco*R1 and *Xba*1 were purchased from Promega Corporation (Madison, WI, USA). The plasmid construct pGem3Z-β_1 encoding for the rat β_1-adrenoceptor was kindly provided by Dr. Curtis A. Machida (Oregon Health and Science University, School of Dentistry, Department of Integrative Biosciences, Portland, OR, USA). Quik change site-directed mutagenesis kit was purchased from Stratagene (La Jolle, CA, USA). (+/–)[^{125}Iodo]cyanopindolol was kindly produced by Mr. David Casley (The University of Melbourne, Department of Medicine, Vic., Australia) using the chloramine-T/NaI method [12]. Glutamine, (+/–)-propranolol, HEPES and polyethyleneimine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). (+/–)Cyanopindolol was obtained from Tocris Cooksen Ltd. (Bristol, UK). All other chemicals were of reagent grade from BDH Chemicals (Kilsyth, Australia).

5.2. Mutagenesis of rat β_1-adrenoceptor cDNA

For receptor expression, the entire coding region derived from pGem3Z-β_1 (base pairs –82 to +1573) was inserted into the *Eco*R1 and *Xba*1 sites of the mammalian vector pcDNA3.1 which provides G418 selection. Site-directed mutagenesis of the codon for the amino acid $Tyr^{356\ (7.43)}$ was performed using the Quik change method according to the manufacturer's instructions. $Tyr^{356\ (7.43)}$ was changed to Phe (Y7.43F) and Ala (Y7.43A). The identities of the mutations were confirmed by automated sequencing on an ABI sequencer.

5.3. Transfection and cell culture

Wild type and mutant plasmids were stably transfected into CHO cells by electroporation. Transfected clones were selected in the presence of 800 μg/ml G418. Colonies originating from single cells were subcloned and evaluated for receptor expression using (+/–)[^{125}Iodo]cyanopindolol binding. Permanent lines of stably expressing cells were maintained in a monolayer culture in Hams F12 media supplemented with 10% foetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and 800 μg/ml G418 in a 10% CO_2 incubator at 37 °C.

5.4. Preparation of crude cell membranes

Preconfluent cells were washed twice with ice-cold phosphate buffer solution and harvested with 25 mM Tris–HCl, pH 7.5, 1 mM EDTA buffer. Harvested cells were homogenised with 20 strokes of a Dounce pestle and then centrifuged at 10,000 × *g* for 15 min. The final membrane pellets were resuspended in Hank's balanced salt solution and stored at –80 °C until needed [13]. Cells were thawed as required and resuspended in Hank's balanced salt solution supplemented with 20 mM HEPES and 0.1% (mass/vol) BSA at 100–200 μg protein/ml for all studies. Protein content was determined by the Bradford method [14] using BSA as the standard.

5.5. Radioligand binding studies

Binding studies were conducted as previously described [13]. For inhibition assays, cell membranes were incubated with 100 pM (+/–)[^{125}Iodo]cyanopindolol and various concentrations of (+/–)cyanopindolol, in a final volume of 0.2 ml. Membranes were incubated for 1 h at 37 °C in the dark and the assay terminated by rapid filtration over glass fibre filters presoaked with (0.1%) polyethyleneimine. For saturation studies, 0.1 ml of membranes were incubated in triplicate with nine different concentrations of (+/–)[^{125}Iodo]cyanopindolol (2 pM–1 nM). Non-specific binding was determined in the presence of 10 μM propanolol.

5.6. Data analysis

The data was expressed as the mean ± standard error of the mean (SEM) of 3–5 independent experiments. Dissociation constant (K_d) (saturation studies), inhibition constant (K_i) (drug inhibition) and receptor binding density (B_{max}) values were determined using the computerised iterative curve fitting program EBDA version 4.0 which incorporates LIGAND version 4.0 [15,16]. Pseudo Hill coefficients (n_H) were obtained from analysis of binding data using the sigmoidal fit function of the EBDA program. Student's *t* test was used to evaluate differences between means and critical values of $P < 0.05$ were judged significant.

5.7. Homology modelling of the rat β_1-adrenoceptor

A three-dimensional homology model of the seven tm regions of the rat β_1-adrenoceptor was constructed using the crystal structure of inactive bovine rhodopsin as a template (PDB file 1F88) [7], hence the rat β_1-adrenoceptor model was also presumed to be in an inactive conformation. Loop regions were not included in the model. The seven tm sequences of the rat β_1-adrenoceptor and bovine rhodopsin were aligned (not shown here, alignment is consistent with Palczewski et al. [7]). The amino acid sequence of the bovine rhodopsin crystal structure was mutated to that of the rat β_1-adrenoceptor using the mutate functionality in the Biopolymer module of the modelling program SYBYL (version 6.8, Tripos Assoc Inc., St. Louis, USA). In tm1 of bovine rhodopsin $Ala^{1.36}$ was excised from the helix in order to maintain the alignment of the conserved $Trp^{1.30}$, $Met^{1.34}$, $Leu^{1.41}$ and $Leu^{1.52}$ residues located at the extracellular end of the helix. As yet we do not know the role played by these conserved residues in G-protein coupled receptors but felt it was important to maintain their alignment. The resulting rat β_1-adrenoceptor tm1 was annealed in the region of the excision site and compared to the bovine rhodopsin tm1 to confirm that conserved residues were still in alignment. Excision or inclusion of $Ala^{1.36}$ in the construction of the rat β_1-adrenoceptor model does not affect our modelling results as the extracellular end of tm1 does not appear to be involved in the ligand binding site. The crude rat β_1-adrenoceptor model was then subjected to molecular mechanics minimisation using the following parameters; Kollman all atom force field and atomic charges, conjugate gradient minimisation, distance dielectric function and a dielectric constant of 5.0, non-bonded cut off 8 Å and 2000 maximum iterations (the same molecular mechanics parameters were used when annealing the section of tm1 described above). After minimisation the rat β_1-adrenoceptor model was superimposed onto the bovine rhodopsin crystal structure via the backbone atoms and the resulting RMS was 0.8 Å. The rat β_1-adrenoceptor model was also checked for consistency with the known experimental data, predominantly β_2-adrenoceptor mutation data [1–5,8–11].

For hydrogen bond interactions, donor–acceptor distances of 3.0–4.0 Å were considered to be feasible. Distances of 4.0–7.0 Å between ring centroids were deemed acceptable for aromatic–aromatic interactions.

In this study, most of the amino acid residues have been dually numbered. In superscript they are numbered according to their position in the receptor sequence and in brackets they have been given a number which corresponds to their relative position in the helix [17]. In this universal numbering system, every amino acid number begins with the tm number, followed by a number which defines their position relative to the most conserved residue in that tm. The reference residue is assigned the number 50.

5.8. Docking (S)-cyanopindolol and (S)-[^{125}Iodo] cyanopindolol into the rat β_1-adrenoceptor model

The antagonists (*S*)-(–)-cyanopindolol and (*S*)-(–)-[^{125}Iodo]cyanopindolol were constructed within the sketch functionality of SYBYL in a fully extended conformation. The (*S*)-isomers were used as they are known to be the most active isomers. Both compounds were structurally optimised using the Tripos molecular mechanics force field and Gasteiger–Huckel atomic charges (all other parameters were left at the default values), and then using the AM1 Hamiltonian within the MOPAC (version 6.00) program as supplied with SYBYL. The parameters used for the semi-empirical molecular orbital calculations were as follows; AM1 Hamiltonian, precise convergence, no molecular mechanics correction for amide linkages and full geometry optimisation.

The individual antagonists were then manually docked into the rat β_1-adrenoceptor ligand binding site, allowing torsion angles to vary in order to maximise the antagonist interactions with $Asp^{138\ (3.32)}$ and $Asn^{352\ (7.39)}$ (described in the Section 1). Once the compound was docked into the binding site, the entire antagonist/receptor complex was subjected to molecular mechanics minimisation following the same protocol as for the model construction, with the exceptions that the Tripos force field, Gasteiger–Huckel atomic charges and 1000 maximum iterations were used. For each antagonist several different binding modes were examined.

The antagonist molecules were extracted out of the minimised antagonist/receptor complex and the ΔH_f^0 was calculated for the bound antagonist conformation using the AM1 Hamiltonian within MOPAC. The parameters for the semi-empirical molecular orbital calculations were the same as described above for the fully extended antagonist conformations, except the torsion angles were not optimised in the bound conformations. For both antagonists, the ΔH_f^0 for the various bound conformations were compared to the ΔH_f^0 of the fully extended conformation to ensure that the bound conformation was energetically feasible (i.e. within 10 kcal/mol of the fully extended conformation and bound conformations with a ΔH_f^0 more than 10 kcal/mol higher were considered to be unrealistic and discarded).

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