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Structure-affinity profile of 8-hydroxycarbostyril-based agonists that dissociate slowly from the β_2-adrenoceptor

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Abstract Several carbostyril-based β-agonists have been shown to bind tightly to and slowly dissociate from the β_2-adrenoceptor (β_2AR). In the present study, the structural features of 8-hydroxy-5-[2-[(1-phenyl-2-methylprop-2-yl)amino]-1-hydroxyethyl]-carbostyril (11a) which contribute to its binding properties at the β_2AR were investigated using a series of synthesized analogs. The k_{off}, estimated by the rate of cAMP decline in DDT_1 MF-2 (DDT) cells with a reduced receptor density, K_i and ligand-induced receptor reductions were determined. All of the derivatives stimulated cAMP accumulation in DDT cells in the sub to mid nanomolar range and elicited the same maximal stimulation as (–)isoproterenol. Derivatives of 11a with side chain *N*-substitutions comprising 2-methylbutyl, phenylethyl and isopropyl had higher k_{off}-values and lower affinities as compared to 11a. Increasing the number of methylenes between the side chain tertiary alpha carbon and phenyl from 1 in 11a to 3 or reducing the number to 0 also resulted in derivatives with higher k_{off}- and K_i-values. In addition, replacement of the 8-hydroxycarbostyril nucleus of 11a with catechol reduced the affinity of the compound for the β_2AR by 48-fold and increased its k_{off}. Only those derivatives with the lowest k_{off}-values induced a decrease in the receptor density of DDT cell membranes following a preincubation and extensive washing. The data show that the 8-hydroxycarbostyril nucleus in conjunction with substitutions on the tertiary alpha carbon of the side chain and positioning of the phenyl group are important characteristics determining the high affinity and slow dissociation of 11a from the β_2AR.

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Introduction

Insurmountable receptor antagonists are characterized by producing a reduction in maximal responses to agonists with or without a change in agonist potency. In terms of receptor-ligand interactions, this type of antagonism usually occurs by covalent irreversible binding of the ligand to, or slow dissociation from, the receptor although additional mechanisms have been proposed (Kenakin 1984; Bond et al. 1989). Several noncovalent insurmountable antagonists for the β-adrenoceptor (βAR) have been reported including FM24 (Lucas et al. 1979), ICI 147,798 (Keith et al. 1989), nebivolol (Pauwels et al. 1988), teratolol (De Blasi et al. 1988) and celiprolol (Doggrell 1990) and these appear to tightly bind to, and slowly dissociate from, the receptor. In keeping with their slow dissociation, several of these antagonists have been shown to have a relatively long duration of action in vivo that is independent of their plasma levels (Le Fur et al. 1980; Keith et al. 1989).

In 1976, a series of β-agonists based upon 8-hydroxycarbostyril were synthesized which subsequently lead to the development of the selective β_2-agonist procaterol (Yoshizaki et al. 1976, 1977). Because some of these agonists showed high potency for stimulating βAR responses and good chemical stability, additional 8-hydroxycarbo-styril derivatives containing side chain phenyl substituents were synthesized by Milecki et al. (1987). One of these derivatives, 5-[2-[[3-[4-(bromoacetamido)phenyl]-2-methyl-prop-2-yl]amino]-1-hydroxyethyl]-8-hydroxycarbostyril (carbo-Br), contained a reactive bromoacetamide moiety in the para position of the side chain phenyl ring which after apparent covalent binding to the receptor acted as an irreversible β-agonist. However, 5-[2[[1-(4-aminophenyl)-2-methylprop-2-yl]amino]-1-hydroxyethyl]-8-hydroxycarbostyril (carbo-amine), another derivative which contained a

para amino substitutent on the side chain phenyl ring, was a potent β-agonist which produced antagonist-insensitive activation of adenylyl cyclase activity in rat reticulocyte membranes (Standifer et al. 1989). Furthermore, pretreatment of reticulocyte membranes with carbo-amine reduced the βAR content which was not reversed by extensive membrane washing but recovery of the receptors occurred by incubation of the membranes at elevated temperatures. This data indicated that carbo-amine bound to the βAR in a noncovalent manner and slowly dissociated from it. It is well known that β-agonists tightly bind to the βAR during ternary complex formation consisting of the agonist, receptor and a stimulatory guanine nucleotide binding protein (G_s). In the presence of a guanine nucleotide, the complex is destabilized and agonist affinity is greatly reduced mainly by an increase in dissociation rate (Williams and Lefkowitz 1977; De Lean et al. 1980; Heidenreich et al. 1980). However, the tight binding of carbo-amine to the βAR appeared to be independent of ternary complex formation because both the functional and binding assays were performed in the presence of a guanine nucleotide to maintain the receptor in the agonist low affinity state (Standifer et al. 1989). More recently TA 2005, an 8-hydroxycarbostyril derivative related to carbo-amine, was shown by radioligand binding assays and in isolated tissue studies to also tightly bind to the βAR (Voss et al. 1992). Although a number of studies have characterized potent and selective β-agonists, including several using the 8-hydroxycarbostyril (Yoshizaki et al. 1976, 1977; Hieble 1991; Ruffolo et al. 1995), the chemical structures of 8-hydroxycarbostyril-based β-agonists that contribute to high affinity binding and/or slow dissociation have not been defined.

In the present report, a structure-affinity profile for a series of β-agonists was undertaken in order to elucidate the ligand structural requirements that contribute to high affinity for and slow dissociation from the β_2AR. The agonists synthesized consisted of 8-hydroxycarbostyril derivatives with varying substituents on the side chain amino group. In addition, the contribution of the 8-hydroxycarbostyril nucleus to slow dissociation was investigated by replacement with a catechol ring. Using DDT_1 MF-2 (DDT) cells which express the β_2AR (Norris et al. 1983), the agonist structures are related to their apparent dissociation rates (indirectly estimated by the rate of cAMP decline), inhibition binding constants (K_is) and by ligand-induced receptor reductions determined by radioligand binding.

Materials and methods

Chemical methods and synthesis

Analytical procedures

Melting points were determined on a Fisher Jones melting point apparatus and are uncorrected. ^{1}H NMR spectra were obtained on a QE-300 MHz spectrometer (300 MHz, FT mode) with Me_4Si as the internal standard. ^{13}C NMR spectra were obtained using a QE (75 MHz, FT mode) spectrometer. Column chromatography and preparative TLC were carried out using silica gel (Merck 70–230 mesh) and Analtech 20×20 cm plates, 2000 microns, respectively. High resolution mass spectra were obtained using a Fennigan MAT 95Q.

Synthesis of β-agonists

The synthetic scheme for the 8-hydroxycarbostyril derivatives is shown in Fig. 1. Synthesis of epoxide 9 was performed using a modified procedure originally described by Milecki et al. (1987). Commercially available 8-hydroxyquinoline was oxidized quantitatively to 8-hydroxyquinoline *N*-oxide 1 which was converted in a two-step sequence to 8-hydroxycarbostyril 3 with excellent yield. Substitution at the 5-position of the aromatic ring with glyoxylic acid, followed by further functionalization, led to 8-benzyloxy-5-oxiranyl carbostyril 9 that served as the starting material for epoxide ring opening reactions with the selected amines. All final products were racemates.

General procedure for preparation of amine adducts (10a–10g; Fig. 1)

Epoxide 9 and the corresponding amine were heated neat or in *n*-butanol for 4 h. The solvent was then removed under vacuum, the residue was dissolved in methylene chloride and extracted with HCl solution (1%). The organic layer was washed with water, dried ($MgSO_4$) and the solvent removed. Final products were obtained by purification of the organic extracts on silica gel column using methylene chloride/methanol with an increasing gradient of methanol. 1,1-Dimethylbenzylamine was synthesized using a previously published procedure (Balderman and Kalir 1978). 2-Amino-2-methyl-5-phenylpentane was synthesized according to the method described by Christol et al. (1961).

8-(Benzyloxy)-5-[2-(1-phenyl-2-methylprop-2yl)-amino]-1-hydroxyethyl carbostyril (*10a*). All structural data are in agreement with those previously published (Milecki et al. 1987).

2-{2-Hydroxy-2-[8-benzyloxy-2(1H)-quinolinonyl]-ethylamino}propane (*10b*). The yield was 0.91 g (76%). M.p. 223–225°C. Found: C 64.65, H 6.56, N 7.11. For $C_{21}H_{24}N_2O_3$×HCl required: C 64.87, H 6.46, N 7.20. ^{1}H NMR (MeOD): 1.02 (3H, t), 1.75–1.83 (2H, m), 3.02–3.08 (2H, t), 3.19–3.22 (2H, m), 5.28 (2H, s), 5.42–5.55 (1H, m), 6.65 (1H, d), 7.18 (1H, d), 7.25–7.38 (3H, m), 7.46 (2H, d), 8.42 (1H, d). ^{13}C NMR ($CDCl_3$ + MeOD): 11.28, 20.47, 50.62, 67.15, 71.80, 113.37, 118.68, 122.02, 122.51, 128.80, 129.29, 129.65, 130.34, 130.93, 137.53, 138.63, 142.37, 143.10, 143.21, 146.04, 162.00. HRMS 353.1816 (M + H). $C_{21}H_{25}N_2O_3$ requires 353.1865.

2-{2-Hydroxy-2[8-benzyloxy-2(1H)-quinolinonyl]-ethylamino}-2-methylbutane (*10c*). The yield was 76%. Product was recrystallized from absolute ethanol. M.p. 210–212°C. Found: C 72.96, H 7.92, N 7.25. For $C_{23}H_{28}N_3O_3$ required: C 72.60, H 7.42, N 7.36. ^{1}H NMR (DMSO): 0.90 (3H, t), 1.30 (6H, s), 1.65–1.75 (2H, m), 2.90–3.10 (2H, m), 3.40–3.60 (2H, m), 5.35 (2H, s), 5.55–5.65 (1H, d), 6.61 (1H, d), 7.21–7.40 (5H, m), 7.60 (2H, d), 8.45 (1H, d), 10.85 (1H, broad signal). ^{13}C NMR (DMSO): 8.33, 22.31, 22.40, 30.25, 48.56, 59.82, 65.64, 70.15, 112.56, 116.93, 119.89, 122.92, 128.20, 128.30, 128.75, 129.81, 130.85, 136.86, 137.02, 144.17, 161.35. HRMS 381.2188 (M + H). $C_{23}H_{28}N_3O_3$ requires 371.2178.

5-[2-(N-Phenethyl)-1-hydroxyethylamino]-8-benzyloxy-2(1H)-quinolinone (*10d*). The yield was 47%. M.p. 100–103°C. Found: C 75.10, H 6.53, N 6.71. For $C_{26}H_{26}N_2O_3$ required: C 75.34, H 6.32, N 6.76. ^{1}H NMR (MeOD): 2.78–2.98 (6H, m), 5.09 (2H, s), 5.18–5.20 (1H, dd), 6.55 (1H, d), 6.92 (1H, d), 7.12–7.26 (6H, m), 7.33–7.39 (5H, m), 8.08 (1H, d).

5-{2-[2-(Phenylprop-2-yl)amino]-1-hydroxyethyl}-8-benzyloxy-2(1H)-quinolinone (*10e*). The yield was 0.8 g (63%). M.p. 239°C (decomposition). ^{1}H NMR (DMSO): 1.82 (6H, d), 2.75–2.90 (2H, m), 5.30

(2H, s), 5.32–5.36 (1H, m), 6.55 (1H, d), 7.10–7.50 (10H, m), 7.60–7.65 (2H, m), 7.92 (1H, d). HRMS 429.2188 (M + H). $C_{27}H_{29}N_2O_3$ requires 429.2178.

5-[2-(2-Methyl-5-phenylpent-2-yl)amino-1-hydroxyethyl]-8-benzyloxy-2(1H)-quinolinone (*10f*). The yield was 0.9 g (64%). Recrystallization from 2-propanol gave analytically pure product. M.p. 224–225°C. ^{1}H NMR (DMSO): 1.32 (6H, s), 1.68 (4H, m), 2.59 (2H, m), 2.90 (2H, m), 5.40 (2H, s), 5.60 (1H, m), 6.25 (1H, s), 6.63 (1H, d), 7.22–7.42 (9H, m), 7.63 (2H, d), 8.47 (1H, d), 9.80 (1H, broad signal), 10.8 (1H, s). ^{13}C NMR (DMSO): 26.63, 24.93, 35.21, 36.63, 48.03, 59.02, 65.14, 69.67, 112.14, 116.48, 119.46, 122.52, 125.78, 127.78, 127.90, 128.25, 128.35, 129.40, 130.37, 136.35, 136.60, 141.61, 143.74, 160.94. HRMS 471.2641 (M + H). $C_{30}H_{35}N_2O_3$ requires 471.2648.

5-[2-(2-Propylamino)-1-hydroxyethyl]-8-benzyloxy-2(1H)-quinolinone (*10g*). The yield was 0.48 g (65.5%). M.p. 122–123°C. HRMS 353.1895 (M + H). $C_{21}H_{25}N_2O_3$ requires 353.1865.

General procedure for preparation of ligands (11a–11fg; Fig. 1)

The protected aminoalcohols (10a–10f) were dissolved in methanol and ammonium formate (excess) followed by palladium on charcoal (10%) added at once under nitrogen. The reaction was heated until no more starting material was detected. The mixture was then cooled to room temperature, filtered through celite and the filtrate concentrated under vacuum. Products were purified using preparative thin layer chromatography on silica gel using a mixture of methylene chloride-methanol-ammonium hydroxide as eluent unless specified otherwise.

8-(Hydroxy)-5-[2-(1-phenyl-2-methylprop-2yl)-amino]-1-hydroxyethyl] carbostyril (*11a*). All structural data are in agreement with those published by Milecki et al. (1987).

2-{2-Hydroxy-2[8-hydroxy-2(1H)-quinolinonyl]-ethylamino}propane (*11b*). Crude product was purified by recrystallization from ethanol-ethyl acetate. The yield was 1.06 g (75%). M.p. 235°C. ^{1}H NMR (DMSO): 0.91 (3H, t), 2.42–2.61 (2H, m), 3.65–3.80 (4H, m), 5.43–5.46 (1H, m), 6.55 (1H, d), 7.02 (1H, d), 7.16 (1H, d), 8.29 (1H, d). HRMS 263.1326 (M + H). $C_{14}H_{19}N_2O_3$ requires 263.1395.

2-{2-Hydroxy-2[8-hydroxy-2(1H)-quinolinonyl]-ethylamino}-2-methylbutane (*11c*). The yield was 68%. M.p. 176°C. ^{1}H NMR ($CDCl_3$ + MeOD): 1.01 (3H, t), 1.38 (6H, d), 1.20–1.35 (2H, m), 3.10–3.35 (4H, m), 5.52 (1H, d), 6.65 (1H, d), 7.05 (1H, d), 7.35 (1H, d), 8.45 (1H, d). ^{13}C NMR ($CDCl_3$ + MeOD): 6.90, 21.52, 21.64, 23.67, 30.23, 32.65, 60.15, 65.92, 113.84, 117.55, 120.82, 128.19, 128.19, 137.38, 144.0, 162.59. HRMS 291.1679 (M + H). $C_{16}H_{23}N_2O_3$ requires 291.1709.

5-[2-(N-Phenethyl)-1-hydroxyethylamino]-8-hydroxy-2(1H)-quinolinone (*11d*). The yield was 85%. The product was purified by column chromatography on silica gel using 5–7% methanol in methylene chloride as eluting system. M.p. 132–134°C. ^{1}H NMR (MeOD): 2.80–2.98 (2H, m), 3.01–3.08 (2H, m), 3.31 (2H, M), 5.10–5.15 (1H, m), 6.80 (1H, m), 6.90–7.0 (1H, m), 7.05–7.17 (6H, m), 8.25–8.40 (1H, m). HRMS 325.1552 (M + H). $C_{19}H_{21}N_2O_3$ requires 325.1551.

5-{2-[2-(Phenylprop-2-yl)amino]-1-hydroxyethyl}-8-hydroxy-2(1H)-quinolinone (*11e*). The crude product was purified on preparative TLC using 15% methanol in methylene chloride. The yield was 170 mg (71%). M.p. 160–162°C. ^{1}H NMR (DMSO): 1.8 (6H, d), 2.75–2.85 (2H, m), 6.50 (1H, d), 6.95 (1H, d), 7.15 (1H, d), 7.38–7.50 (3H, m), 7.60–7.65 (2H, m), 7.9 (1H, d), 8.50 (1H, m). HRMS 339.1730 (M + H). $C_{20}H_{23}N_2O_3$ requires 339.1708.

5-[2-(2-Methyl-5-phenylpent-2-yl)amino-1-hydroxyethyl]-8-hydroxy-2(1H)-quinolinone (*11f*). Yield was 0.12 g (63%). M.p. 122–124°C. ^{1}H NMR (DMSO) 1.23 (6H, s), 1.62 (4H, s), 2.50–2.60 (2H, m), 2.82–2.98 (2H, m), 5.40 (1H, d), 6.0–7.0 (broad signal), 6.60 (1H, d), 7.10–7.30 (6H, m), 8.25 (1H, d), 8.50 (1H, s). ^{13}C NMR (DMSO) 23.16, 25.09, 35.41, 37.10, 48.50, 58.20, 65.75, 114.25, 116.86, 120.0, 121.92, 125.87, 128.36, 128.58, 128.65, 136.81, 141.79, 144.14, 161.05, 166.79. HRMS 381.2146 (M + H). $C_{23}H_{29}N_2O_3$ requires 381.2178.

5-[2-(2-Propylamino)-1-hydroxyethyl]-8-hydroxy-2(1H)-quinolinone (*11 g*). The yield was 150 mg (71%). M.p. 145–149°C. ^{1}H NMR (MeOD): 1.34 (6H, s), 3.19 (2H, s), 3.40–3.44 (1H, m), 5.42 (2H, s), 6.55–6.57 (1H, m), 6.99–7.05 (1H, m), 7.23–7.25 (1H, m), 8.27–8.29 (1H, m), 8.53 (1H, s). ^{13}C NMR (MeOD): 19.0, 52.0, 67.36, 67.44, 115.40, 118.96, 122.16, 129.29, 129.35, 129.40, 138.66, 145.48, 163.85. HRMS 263.1391 (M + H). $C_{14}H_{19}N_2O_3$ requires 263.1395.

Synthesis of catecholamine derivative (Fig. 2)

The synthetic scheme for the catecholamine epoxide 12 was performed according to the procedure described by Marki et al. (1988). The epoxide was reacted neat with phenteramine and the adduct purified by column chromatography. In the final step, the two benzyl protecting groups were removed by hydrogenolysis using 1,4-cyclohexadiene as hydrogen source.

1-{[2-[(1-Phenyl-2-methylprop-2-yl)amino]-1-hydroxyethyl]-3,4-bis(benzyloxy)}-benzene (*13*). 1,2-Bis(benzyloxy)-4-oxiranylbenzene 12 (2.89 g, 9.4 mmol) was reacted with freshly prepared phenteramine (3 ml, excess) and the mixture was heated under nitrogen for 6 h. The reaction was monitored by TLC, and when epoxide was no longer detected, the mixture was diluted with methylene chloride (50 ml) and extracted with HCl (1%) to remove unreacted amine. The organic layer was washed with water and dried ($MgSO_4$). After the solvent was removed the crude material was purified on silica gel column chromatography using 2–5% methanol in methylene chloride as eluent. The yield was 1.84 g (43%). M.p. 121–122°C. ^{1}H NMR ($CDCl_3$): 1.03 (3H, s), 1.05 (3H, s), 2.56 (2H, s), 2.58–2.68 (2H, m), 2.82–2.87 (1H, q), 4.49–4.53 (1H, m), 5.14 (2H, s), 5.16 (2H, s), 5.42–7.73 (18H, m). ^{13}C NMR ($CDCl_3$): 27.62, 26.68, 49.94, 54.08, 57.90, 67.23, 71.55, 71.64, 119.94, 126.14, 127.69, 127.72, 127.82, 128.37, 130.59, 137.35, 138.32. HRMS (M + H) 482.2695. For $C_{32}H_{36}NO_3$ required 482.2660.

1-{[2-[(1-Phenyl-2-methylprop-2-yl)amino]-1-hydroxyethyl]-3,4-bis(hydroxy)}-benzene (*14*). Adduct 13 (100 mg, 0.2 mmol) was dissolved in 10 ml methanol. Catalyst (10% Pd/C, 80 mg) was added followed by 1,4-cyclohexadiene (0.2 ml, 2 mmol). This mixture was refluxed until the starting material was no longer detected by TLC. The suspension was filtered through celite and then concentrated yielding a yellow solid (33.8 mg, 56%). M.p. 102–105°C (decomposition). ^{1}H NMR (MeOD): 1.09 (6H, s), 2.75–2.95 (2H, m), 3.34–3.63 (2H, m), 4.51–4.60 (1H, m), 6.73–6.89 (5H, m), 7.13–7.29 (5H, m). HRMS (M + H) 302.1755. For $C_{18}H_{24}NO_3$ required 302.1756.

Biological methods

Cell culture

DDT cells were grown as monolayers on poly-L-lysine-treated 150-mm plastic petri dishes. The dishes were prepared by adding 10 ml sterile water containing 0.01 mg/ml poly-L-lysine and after 1 h at room temperature they were rinsed with 2×5 ml water. Cells were grown in 30 ml Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 100 U/ml penicillin G, 2.5 µg/ml amphotericin B, and 0.1 mg/ml streptomycin sulfate in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were subcultured twice weekly by detachment in 10 ml Hank's Balanced Salt Solution (HBSS) without divalent cations and containing 1 mM EDTA. Cells were seeded at 2–5×10^5 per dish and experiments performed on 1 day preconfluent cultures.

Membrane preparation and pretreatments

Attached cells were washed twice with 10 ml HBSS, scraped free of the dish in 4 ml of 50 mM Tris-HCl buffer, pH 7.4 at 4°C, and the suspension homogenized with a Tekmar homogenizer at setting 20 for 10 s. The suspension was then diluted to 35 ml in homogenization buffer and centrifuged at 27,000 *g* for 10 min. The membrane pellet was resuspended in 4 ml homogenization buffer by vortexing and centrifuged as before. The final pellet was resuspended in 50 mM Tris-HCl buffer at pH 7.4 containing 5 mM $MgCl_2$ for assays. Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

In pretreatment experiments, DDT cell membranes (1 mg/ml protein) were incubated in 50 mM Tris-HCl buffer at pH 7.4 containing 100 µM 5'-guanylylimidodiphosphate [Gpp(NH)p] and without or with 1 µM of the β-agonists for 4 min at 37°C. At the end of the incubation, the suspensions were diluted to 35 ml with ice-cold incubation buffer and centrifuged at 27,000 *g* for 10 min. The pellets were resuspended in 35 ml of ice-cold buffer and centrifuged as before. These wash cycles of resuspension and centrifugation were performed a total of six times and the final pellets were resuspended in 0.5 ml of 50 mM Tris-HCl buffer at pH 7.4 containing 5 mM $MgCl_2$ for assays.

Receptor binding assay

βARs were determined by specific [^{125}I]iodocyanopindolol (CYP) binding. Briefly, cell membranes (5–15 µg protein) were incubated in a total volume of 0.25 ml containing 50 mM Tris-HCl buffer at pH 7.4 containing 5 mM $MgCl_2$, 100 µM Gpp(NH)p, 6–100 pM [^{125}I]CYP and with and without 1 µM (–)alprenolol for 60 min at 36°C. At the end of the incubation, each suspension was diluted with 3 ml of ice-cold incubation buffer and the contents filtered through Whatman GF/B glass fiber filters using a Brandel cell harvester. The filters were washed with an additional 6 ml of ice-cold buffer and the radioactivity retained on the filters was counted in a gamma counter. Specific binding to the βAR was calculated as the difference in total binding determined in the absence of (–)alprenolol and the nonspecific binding determined in the presence of 1 µM (–)alprenolol. Each assay was performed in triplicate and specific binding was usually 80–90% of the total binding.

In experiments to determine the concentration of ligands that inhibited specific [^{125}I]CYP binding by 50%, the assays were the same as above except the [^{125}I]CYP concentration was 30 pM and varying concentrations of the synthesized ligands were included.

cAMP determinations

Attached cells were gently washed twice with 10 ml HBSS, gently detached in 5 ml HBSS using a rubber policeman and the suspension was centrifuged at 500 *g* for 5 min. The cell pellet was resuspended in 1 ml HBSS and aliquots of this suspension (0.1 mg protein) were then preincubated in 0.4 ml HBSS for 5 min at 37°C. At the end of this incubation, rolipram (50 µM) and the β-agonists were added and the incubation continued for the times indicated in the text. The suspensions were then placed in a boiling water bath for 5 min, cooled to room temperature and centrifuged at 13,000 *g* for 2 min. The cAMP content of the supernatants was determined using the radioimmunoassay procedure described in detail by Belardinelli et al. (1996).

The estimated dissociation rate of the β-agonists was determined by the rate of cAMP decline in DDT cells after reducing the βAR density. Attached cells were rinsed twice with 2×10 ml HBSS and incubated for 20 min at 37°C in 10 ml HBSS containing 1 nM 5[2-[[[1'-(4'-isothiocyanatophenyl)thiocarbonyl]-amino]-2-methylpropyl]amino-2-hydroxypropoxy]-3,4-dihydrocarbostyril (DCITC), a potent irreversible antagonist for the βAR (Deyrup et al. 1998). At the end of the incubation, the cells were washed 15 times by the addition of ice-cold HBSS (10 ml) followed 5 min later by aspiration to remove the buffer. After the final wash, the cells were detached as described above. Aliquots of the cell suspension were incubated in 0.4 ml HBSS containing 1 µM of the agonist for 4 min at 37°C. At the end of this incubation, propranolol was added to give a concentration of 20 µM and the incubation was continued. At varying times after the addition of propranolol (to prevent agonist rebinding to the receptor), isobutylmethylxanthine (IBMX, 0.1 ml) was added (0.5 mM) and the tubes were placed in a boiling water bath for 5 min. The suspensions were then cooled to room temperature, centrifuged at 13,000 *g* for 2 min. The cAMP content of the supernatants was determined as described above. Using a radioligand competition assay (see receptor binding assay above), the K_i for propranolol for the β_2AR of DDT cells is 2.2±0.6 nM (mean ±SEM). Therefore, the ratio of the K_i for propranolol to the concentration used (20 µM) to determine the agonist k_{off}-values is 9090 which is considerably greater than the ratio (625) of the K_i (see results Table 2) and concentration of 11a used (1 µM). This indicates that the excess of propranolol added was enough to occupy the β_2AR and prevent agonist rebinding after it dissociated from the receptor.

In some experiments, the intracellular and extracellular cAMP content was determined. At the end of the incubation to stimulate cAMP accumulation as above, IBMX was added to a final concentration of 0.5 mM and the cells were centrifuged at 13,000 *g* for 2 min. The supernatant was saved for the determination of extracellular cAMP. The cell pellet was resuspended in 0.5 ml HBSS containing 0.5 mM IBMX, and the suspension placed in a boiling water bath for 5 min. After cooling to room temperature, the tubes were centrifuged at 13,000 *g* for 5 min and the cAMP content of the supernatant (intracellular cAMP) was determined.

Phosphodiesterase activity

DDT cells were homogenized in 40 mM Tris-HCl buffer at pH 8.0 and the phosphodiesterase activity of the whole homogenate was determined as described by Belardinelli et al. (1995).

Data analysis

The concentration of agonists that stimulated cAMP accumulation by 50% (EC_{50}), the maximal response and the concentration of ligands that inhibited [^{125}I]CYP binding by 50% (IC_{50}) were determined from nonlinear regression analysis using the GraphPad Prism program (GraphPad Software, San Diego, Calif., USA). The value of K_i for the displacement of [^{125}I]CYP binding by each agonist and propranolol was calculated using the Cheng and Prusoff (1973) transformation of the IC_{50}-values. The K_d and maximal binding values (B_{max}) for [^{125}I]CYP were determined from nonlinear regression analysis (GraphPad Prism) of saturation binding data using a one-site hyperbola model and displayed as Rosenthal (1967) plots. The occupancy-response relationship was analyzed by nonlinear regression with a one-site rectangular hyperbola (GraphPad Prism) and the receptor reserve for the maximal response defined as 90% of the maximum was calculated from the fitted curve. The apparent k_{off} for the agonists was determined by fitting the rate of cAMP decline to a monoexponential decay equation using nonlinear regression analysis (GraphPad Prism). Statistical analysis of the data was performed using the Student's *t*-test or analysis of variance with a Dunnett's (Montgomery 1997) post test and differences were considered significant if $P<0.05$.

Materials

DDT cells were obtained from the American Type Culture Collection (Rockville, Md., USA). (–)[^{125}I]CYP (2000–2200 Ci/mmol) and [^{3}H]cAMP (28.2 Ci/mmol) were purchased from Dupont NEN (Boston, Mass., USA). DMEM, HBSS and fetal bovine serum were from GIBCO (Grand Island, N.Y., USA). All other chemicals and reagents were purchased from Sigma Chemical (St. Louis, Mo., USA) or Aldrich Chemical (Milwaukee, Wis., USA).

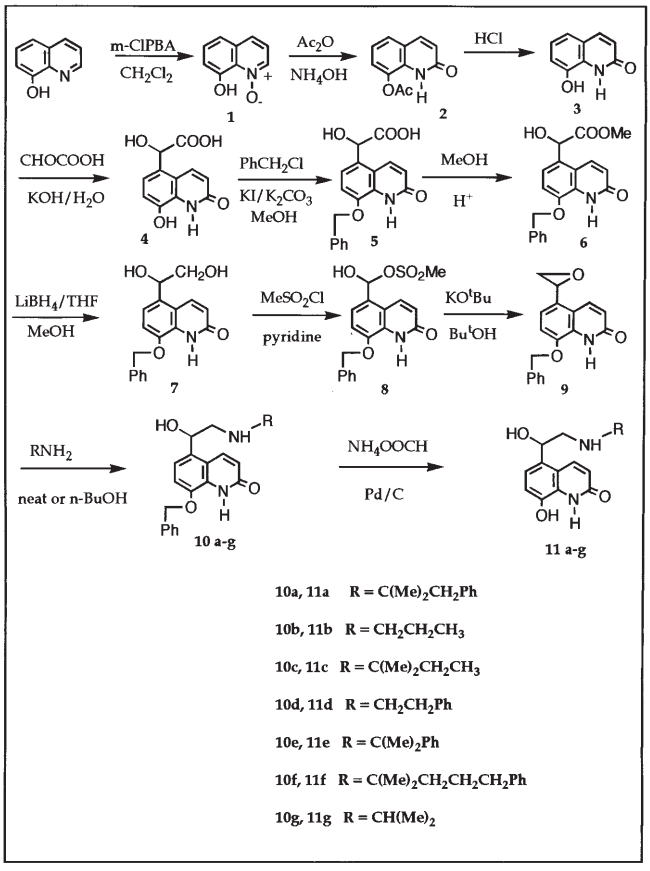


Fig. 1 Synthetic scheme for 8-hydroxycarbostyril derivatives

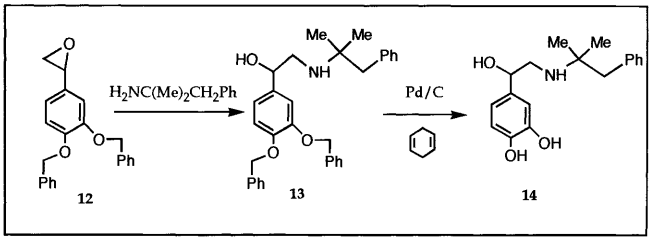


Fig. 2 Synthetic scheme for catecholamine derivative

Results

Compound 11a (Fig. 1) was originally synthesized by Yoshizaki et al. (1976) and reported to be a potent β_2-agonist in isolated tissue preparations. Subsequently, this compound was shown to have a high affinity for the β_2AR in reticulocyte membranes (Standifer et al. 1989). In preliminary experiments, we observed that 11a produced antagonist-insensitive stimulation of cAMP accumulation similar to carbo-amine (unpublished observations). Using 11a as the prototype (Fig. 1), we focused on altering different segments of the side chain phenteramine to assess the effect of these structural modifications on affinity for and slow dissociation from the β_2AR. In derivative 11b, the phenteramine is replaced with propylamine whereas compounds 11c and 11d lack only the two side chain methyl substituents attached to the same carbon atom (geminal methyls) or the phenyl ring, respectively. The number of methylene spacers between the carbon bearing the geminal methyl groups and the phenyl ring was changed to 0 (11e) and 3 (11f). In compound 11g the amino function carries an isopropyl moiety rendering the side chain analogous to (–)isoproterenol (Iso). Finally, in derivative 14 (Fig. 2), catechol replaced the 8-hydroxycarbostyril nucleus of compound 11a to investigate if this ring contributes to slow dissociation.

Table 1 Agonist properties of β-adrenoceptor agonists. DDT cells were incubated with 50 μM rolipram and varying concentrations of the β-agonists for 10 min at 37°C. At the end of the incubation, the cAMP accumulated was determined as described in Materials and methods. Each value is the mean ±SEM with the *n in parentheses*. The basal and Iso maximal stimulation of cAMP accumulation was 63±14 and 1148±74 pmol/mg protein 10 min (*n*=8)

Compound	EC_{50} (nM)	Maximal response (% of (–)isoproterenol maximum)
(–)Isoproterenol	6.2±0.9 (8)	100
11a	0.5±0.1 (9)	104±5
11b	30.7±6.5 (4)	91±4
11c	3.3±1.1 (7)	105±3
11d	14.9±4.5 (4)	105±4
11e	34.8±8.3 (6)	98±12
11f	1.9±0.7 (5)	92±8
11g	5.4±1.6 (8)	97±4
14	6.4±1.6 (7)	92±6

β-Agonist effects of synthesized compounds

The effects of the synthesized compounds and Iso on stimulated cAMP accumulation in DDT cells is shown in Table 1. The EC_{50}-values for cAMP accumulation varied from a low of 0.5 nM for 11a to a high of 34.8 nM for 11e. The overall potency series is 11a>11f>11c>11g=Iso=14>11d >11b=11e. The maximal stimulation of cAMP accumulation for all of the synthesized compounds was similar to that observed by Iso (Table 1). The stimulation of cAMP accumulation produced by 1 μM of all of the agonists was completely blocked by the concurrent addition of 20 μM propranolol. In addition, none of the agonists (1 μM) inhibited phosphodiesterase activity as determined using whole cell homogenates (data not shown).

Apparent dissociation rate (k_{off}) and K_i for the β-agonists

A functional assay was designed to estimate indirectly the apparent k_{off} for each of the β-agonists. In this method, the rate of cAMP decline in DDT cells after stimulation by each agonist alone was determined in the presence of a 20-

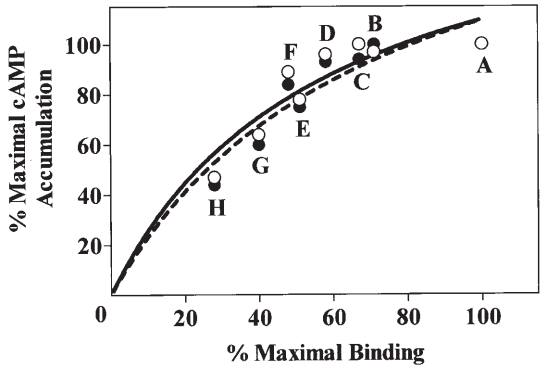
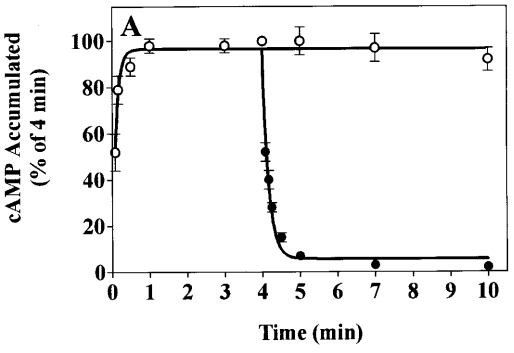


Fig. 3 Relationship between βAR occupancy and maximal response in DDT cells. Attached DDT cells were incubated without (*A*) and with *B* 0.25, *C* 0.5, *D* 0.75, *E* 1.0, *F* 2.5, *G* 5 and *H* 10 nM of the irreversible β-antagonist DCITC for 20 min at 37°C and then washed 15 times in HBSS. Detached cells were then incubated with 50 μM rolipram and without or with 10 μM Iso (○) or 10 μM 11a (●) for 10 min at 37°C. At the end of the incubation, the cAMP accumulated was determined. Parallel samples of DCITC-pretreated cells were homogenized, membranes isolated and the βAR content was determined by the specific binding of 100 pM [^{125}I]CYP. The control Iso- and 11a-stimulated cAMP accumulation was 1264±174 and 1057±150 pmol/mg protein 10 min, respectively (means ±SEM, *n*=4–6). The control [^{125}I]CYP binding was 109±13 fmol/mg protein (means ±SEM, *n*=4–6)



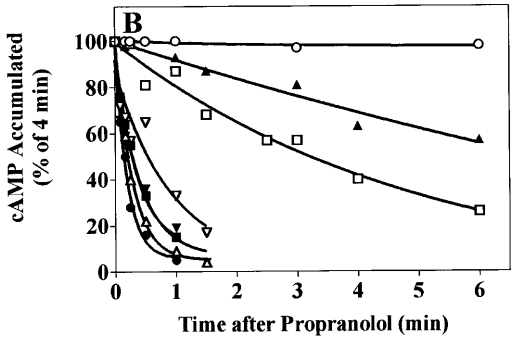


Fig. 4A,B Time course of cAMP accumulation and decline in DDT cells. In panel **A**, suspensions of DDT cells were incubated with 1 μM Iso at 37°C and at the times indicated, the cAMP accumulated was determined (○). After 4 min of incubation, propranolol (20 μM) was added to parallel samples and the cAMP accumulated was measured as a function of time (●). The basal and Iso-stimulated cAMP accumulated at 4 min was 31±8 and 676±35 pmol/mg protein (means ±SEM, *n*=7). In panel **B**, cells were incubated with 1 μM 11a (○), 11b (●), 11c (□), 11d (■), 11e (△), 11f (▲), 11g (▽) or 14 (▼) for 4 min at 37°C. Propranolol (20 μM) was then added (time 0) and the cAMP decline was determined at the times indicated

fold excess of the β-antagonist propranolol. Because cAMP formation depends upon receptor occupancy by the agonists during the initial incubation, the subsequent rate of cAMP decline, likely due to degradation by phosphodiesterases, should be related to the k_{off} of the agonist from the receptor. The accuracy of the relationship between the agonist k_{off} from the receptor and the rate of cAMP decline may partly depend upon the presence of a receptor reserve. If a significant receptor reserve exists, the apparent k_{off} will be overestimated due to a nonlinear relationship between receptor occupancy and response. In order to ensure that cAMP accumulation was related to receptor occupancy, initial experiments were performed to determine the receptor reserve for agonist-stimulated cAMP accumulation. DDT cells were pretreated with varying concentrations of the irreversible β-antagonist DCITC, washed 15 times to remove unbound ligand, and the maximal cAMP accumulation by each agonist was determined. The concentration range of DCITC used (0.25–10 nM) reduced the receptor content from 25% to 78% (as determined by Rosenthal plots of [^{125}I]CYP binding) without a change in the K_d-value for this radioligand (data not shown). As illustrated in Fig. 3, a hyperbolic relationship was observed between receptor occupancy and maximal cAMP accumulation for Iso and 11a. Based upon the fitted hyperbolic curve, the maximal stimulation of cAMP accumulation, defined as 90% of the maximum, occurred at receptor occupancies of 62% and 66% for Iso and 11a, respectively. Therefore, the calculated receptor reserve is 38% for Iso and 34% for 11a. Using the same experimental paradigm, the receptor reserve for 11b, 11c, 11d, 11e, 11f, 11g and 14 are 40%, 42%, 28%, 28%, 18%, 32% and 36%, respectively. Therefore, in experiments to determine the apparent k_{off} for the agonists, the receptor content was decreased 40–50% (to delete the receptor reserve) by initially pretreating the cells with 1 nM DCITC followed by washing.

Figure 4A shows the effect of Iso on cAMP accumulation in DCITC-pretreated DDT cells in the absence of a phosphodiesterase inhibitor. This agonist (1 μM) produced a rapid increase in cAMP accumulation reaching a maximum after 1 min of incubation. The maximum stimulation of cAMP accumulation was maintained within 10% during the next 9 min of incubation. Over the 10-min incubation period, less than 10% of the cAMP accumulated was extracellular and with longer incubation periods the levels of cAMP gradually declined (data not shown). The addition of propranolol (20 μM) after 4 min of incubation with Iso resulted in a rapid decline of cAMP which approached basal levels within 1.5 min. Because of the decline in cAMP with incubation periods greater than 10 min, all subsequent experiments to estimate the k_{off} used an incubation period of 6 min after the addition of antagonist. Figure 4B depicts the rate of agonist-stimulated cAMP decline after the addition

Table 2 Agonist apparent k_{off}, K_i and Hill slope values. The apparent k_{off} was calculated as the rate of cAMP decline in 1 nM DCITC-pretreated DDT cells. After a 4-min stimulation of cAMP accumulation by 1 μM of each agonist, propranolol (20 μM) was added and the cAMP determined as a function of time. The K_i and Hill slope values were determined from displacement of [^{125}I]CYP binding in the presence of 100 μM Gpp(NH)p. Each value is the mean ±SEM with the *n in parentheses* (*ND* not detectable)

Agonist	Apparent k_{off} (min^{-1})	K_i (nM)	Hill slope
(–)Isoproterenol	5.5±0.3 (8)	116.8±2.3 (3)	0.84±0.01 (3)
11a	ND	1.6±0.2 (6)	0.87±0.02 (6)
11b	5.4±0.4 (3)	299.5±32.4 (5)	0.97±0.06 (5)
11c	0.21±0.05 (5)	20.5±2.4 (4)	0.93±0.04 (4)
11d	3.4±0.6 (4)	36.9±1.4 (4)	0.85±0.03 (4)
11e	4.6±0.8 (4)	79.9±15.6 (4)	0.84±0.01 (4)
11f	0.045±0.014 (4)	4.5±0.7 (3)	0.88±0.01 (3)
11g	2.0±0.5 (6)	26.8±3.0 (4)	0.82±0.05 (4)
14	4.1±0.6 (5)	76.4±20.5 (4)	0.88±0.04 (4)

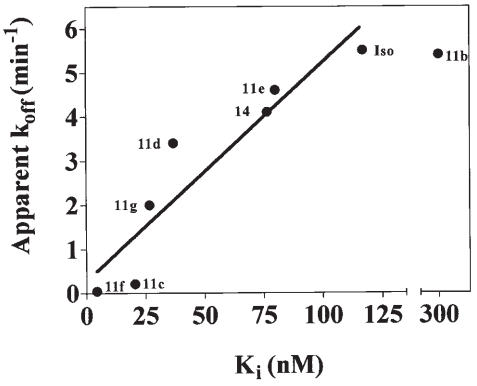


Fig. 5 Linear regression analysis of the relationship between the apparent k_{off} and K_i for the agonists. Data taken from Table 2. The regression line includes all of the agonists except 11b

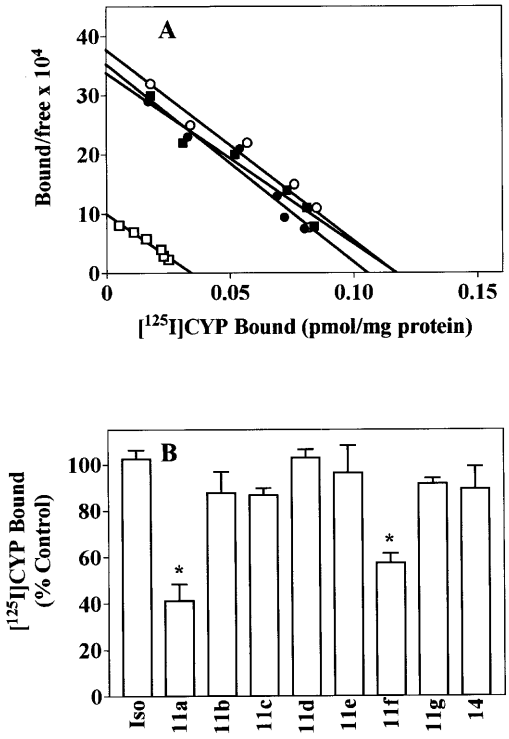


Fig. 6A,B Effect of β-agonist pretreatment on βAR content of DDT cell membranes. In panel **A**, membranes were incubated with 100 μM Gpp(NH)p and without (○) or with 1 μM Iso (●), 11a (□) or 11d (■) for 4 min at 37°C. The membranes were then washed six times and the specific binding of 6–100 pM [^{125}I]CYP (45 min at 36°C) was determined. The data were analyzed by nonlinear regression analysis and displayed as a Rosenthal plot. Data are representative of three separate determinations. In panel **B**, membranes were incubated in the presence of 100 μM Gpp(NH)p and with or without 1 μM of the indicated agonist for 4 min at 37°C. The membranes were then washed six times and the specific binding of 100 pM [^{125}I]CYP (30 min at 36°C) was determined. Data are the means ±SEM of 3–5 separate experiments. The control [^{125}I]CYP binding ranged from 85 to 179 fmol/mg protein. $^*P<0.05$ compared to control using analysis of variance with a Dunnett's post test

of propranolol and Table 2 lists the apparent k_{off} calculated from the rate of cAMP decline. The apparent k_{off} order is Iso=11b>11e>11d>14>11g>11c>11f. An apparent k_{off}-value for 11a could not be determined because no significant cAMP decline was observed during the incubation period after the addition of propranolol (Fig. 4B).

Table 2 also shows the K_i and Hill slope values for the agonists determined from displacement of [^{125}I]CYP binding to the β_2AR in DDT cell membranes in the presence of 100 μM Gpp(NH)p. These K_i-values ranged from a low of 1.6 nM for 11a to a high of 299.5 nM for 11b. The K_i for 11a did not change by increasing the incubation time beyond the 60 min used, indicating that equilibrium binding was approached (data not shown). The Hill slope for the agonists varied from 0.82 to 0.97, suggesting that most of the receptors were in a single affinity state for each agonist. Figure 5 depicts the relationship between the apparent k_{off}- and K_i-values for the agonists. Linear regression analysis using the data from all of the agonists (except 11a) gave an r^2 of 0.51. However, the correlation between the k_{off}- and K_i-values for the agonists was increased (r^2=0.87) when compound 11b was not included in the analysis. Agonist 11b was not included because this agonist had an estimated k_{off} similar to Iso but a higher K_i-value. Assuming similar association rates of Iso and 11b for the β_2AR, then 11b would be predicted to have a higher k_{off} as compared to Iso. Therefore, the lower apparent k_{off} for 11b than expected may be due to this agonist having a faster k_{off} from the receptor than the intrinsic rate of cAMP decline.

Effect of agonists on the β_2AR content of DDT cell membranes

To further investigate if the agonists slowly dissociate from the β_2AR, their effect to reduce maximal [^{125}I]CYP binding

after membrane pretreatment was assessed. As shown in Fig. 6A, pretreatment of DDT cell membranes with 1 μM Iso or 11d in the presence of 100 μM Gpp(NH)p for 4 min at 37°C, followed by six membrane wash cycles, did not alter the B_{max} for [^{125}I]CYP binding to the β_2AR. In contrast, pretreatment with 11a (1 μM, 4 min) reduced maximal [^{125}I]CYP binding by 68%. The K_d for [^{125}I]CYP binding to the β_2AR was not changed after the pretreatment with the agonists followed by membrane washing (control, 25 pM; Iso-pretreated, 24 pM; 11d-pretreated, 30 pM; 11a-pretreated, 25 pM). The lack of change in the K_d-value indicates that the unbound agonists were effectively removed during the membrane washing. Figure 6B shows the effect of the agonists on [^{125}I]CYP binding after a 4-min incubation with DDT cell membranes in the presence of Gpp(NH)p followed by six membrane wash cycles. No change in [^{125}I]CYP binding was observed after pretreatment with Iso, 11b, 11c, 11d, 11e, 11g and 14, indicating that these agonists rapidly dissociated from the β_2AR during the membrane washing. In comparison, a 59% and 43% reduction in [^{125}I]CYP binding occurred after membrane pretreatment with 11a and 11f, respectively. The reduction in [^{125}I]CYP binding after membrane pretreatment with 11a and 11f suggests that a significant fraction of the β_2ARs retain bound agonist for an extended period, which is consistent with 11a and 11f slowly dissociating from the receptor.

Discussion

The goal of the present study was to determine the structural requirements for 8-hydroxycarbostyril-based β-agonists which contribute to high affinity for and slow-dissociation from the β_2AR. Using 11a as the prototype high affinity and slow dissociating agonist, the 8-hydroxycarbostyril nucleus and several side chain substituents were identified as important contributors to the unique binding properties of this compound. All of the derivatives synthesized stimulated cAMP accumulation in DDT cells with potencies ranging from the sub to mid nanomolar range and with a maximal stimulation that was not different from the classical β-agonist Iso. Furthermore, the stimulation of cAMP accumulation by each of the derivatives and Iso was blocked by the concurrent addition of the β-antagonist propranolol. Taken together, these data indicate that the response by each of the compounds is mediated by the β_2AR in DDT cells.

In this study, the agonist structures were related to their estimated dissociation rates (k_{off}), K_i-values for the β_2AR and their ability to induce a decrease in the receptor content of DDT cell membranes. In order to estimate the agonist k_{off} from the β_2AR, a functional assay was employed where the rate of cAMP decline was determined in the presence of excess β-antagonist. Since cAMP accumulation is dependent upon receptor occupancy, the rate of cAMP decline after addition of the antagonist should approximate the agonist k_{off} from the receptor. Cellular functional assays have been previously used to estimate the rate constants for receptor ligands (Sklar et al. 1985; Mueller et al. 1988). However, the estimated k_{off} may be affected by several factors including the presence of a receptor reserve as well as agonist-induced desensitization of the response. In addition, the intrinsic rate of cellular cAMP decline (rate of cAMP degradation) provides the upper limit in estimating high k_{off}-values. A large receptor reserve or nonproportional relationship between receptor occupancy and response could result in an underestimate of the k_{off}. Using the irreversible antagonist DCITC, the agonists' receptor reserve for the maximal stimulation of cAMP accumulation varied from 18% to 42%. Therefore, to achieve a more proportional relationship between receptor occupancy and response and thereby improve the k_{off} estimate, the receptor content (receptor reserve) was reduced by pretreating the cells with the irreversible β-antagonist DCITC (Deyrup et al. 1998). Desensitization of the β_2AR in DDT cells has been reported (Scarpace et al. 1985; Strasser et al. 1986) and may have occurred during the time course that cells were exposed to agonists (10 min). However, the observation that the maximal accumulation of cAMP declined less than 10% indicated that substantial stimulation of cAMP accumulation was sustained during the agonist exposure time. With incubation periods longer than 10 min, desensitization likely became more significant because the maximal cAMP accumulated declined.

The addition of propranolol after initial stimulation of cAMP accumulation with Iso resulted in a rapid decline in cAMP. The estimated k_{off} based upon the rate of cAMP decline was 5.5 min^{-1}. This value is in good agreement with the reported k_{off} (4 min^{-1}) for Iso based upon the recovery of a chemoattractant-mediated cellular response inhibited by βAR activation in neutrophils (Mueller et al. 1988). At the other extreme, the 11a-mediated increase in cAMP accumulation did not decline after the addition of propranolol, indicating that 11a slowly dissociated from the β_2AR. This observation is consistent with that reported for carbo-amine, a β-agonist structurally similar to 11a, which was shown to produce sustained activation of adenylyl cyclase in reticulocyte membranes (Standifer et al. 1989). The antagonist-insensitive effect of 11a was not likely due to non-receptor mediated changes in cAMP accumulation because the increase was blocked by the concurrent addition of propranolol and 11a did not inhibit phosphodiesterase activity at the concentration used. A good correlation between the K_i and the functionally derived k_{off} was obtained for the agonists when 11b was excluded from the analysis. This correlation suggested that the k_{off}-values obtained from the functional assay are a good approximation of the agonists' k_{off} from the receptor. Agonist 11b was excluded from the correlation analysis because its functionally derived apparent k_{off} may be faster than the intrinsic rate of cAMP decline. Compound 11a was also not included in the analysis because little or no cAMP decline occurred after antagonist addition, precluding the calculation of an apparent k_{off}. Nonetheless, 11a had the lowest estimated K_i of the agonists, which is consistent with its sustained binding to the receptor as indicated in the functional assay.

Based upon the estimated k_{off}- and K_i-values for the agonists, several structural characteristics of 11a emerged that are required for its high affinity and slow dissociation from the β_2AR. The relatively high apparent k_{off}- and K_i-values for the side chain amino derivatives comprising propyl (11b), 2-methylbutyl (11c), phenethyl (11d) and isopropyl (11g) indicated that the side chain geminal methyls and phenyl groups are necessary for the slow dissociation observed with 11a. The distance between the geminal methyls and phenyl group also appears to contribute to high affinity and slow dissociation. In 11a, a single methylene separated the geminal methyls and the phenyl group. When the number of methylenes was reduced to 0, as in compound 11e, the estimated k_{off} and the K_i were relatively high. When the number of methylenes was increased to 3 (11f), the estimated k_{off} was relatively low but dissociation was still faster than with 11a. Finally, replacing the 8-hydroxycarbostyril nucleus of 11a with catechol (14) resulted in an agonist with a relatively higher k_{off}- and K_i-value. This suggested that the 8-hydroxycarbostyril nucleus in conjunction with the side chain geminal methyls and phenyl group are contributing factors to the slow dissociation and low K_i of 11a. It has been proposed that the hydroxyl and amido groups of 8-hydroxycarbostyril correspond to the *p*- and *m*-hydroxyl groups of catecholamines, respectively, and may interact with the same serine residues in the β_2AR (Yoshizaki et al. 1976; Kikkawa et al. 1997). Interestingly, replacement of the 8-hydroxycarbostyril of 11a with catechol (14) reduces the affinity of the latter by 48-fold. Because the side chain substituents for both compounds are the same, this would indicate that the 8-hydroxycarbostyril nucleus contributes significantly to the overall affinity of 11a through distinct interactions with the receptor unavailable to catechol. These may include interactions with the carbonyl or enhanced interactions between the receptor and the ring system.

The structure-affinity profile based upon the estimated k_{off}- and K_i-values was largely confirmed using radioligand binding. Thus, pretreatment of DDT cell membranes with 11a and 11f, which have the lowest apparent k_{off}- and K_i-values, produced a decrease in the B_{max} for [^{125}I]CYP binding to the β_2AR. The lack of complete loss of [^{125}I]CYP binding after pretreatment with a concentration of these agonists (1 μM) that occupied greater than 90% of the β_2ARs indicates that some dissociation occurred during the subsequent membrane wash cycles and 30-min incubation with the radioligand. Nonetheless, the decrease in receptor content was likely due to sustained binding of 11a and 11f to the receptor. All of the other agonists, which had higher apparent k_{off}- and K_i-values than 11a and 11f, did not produce a decrease in radioligand binding, indicating that they rapidly dissociated from the receptor. Although our data indicate that the side chain geminal methyls and phenyl group are involved in the ligand structural requirements for slow dissociation, other side chain modifications may also suffice. For example, TA 2005, another 8-hydroxycarbostyril-based β-agonist which contains a single methyl on the alpha-carbon to the amino group and a *p*-methoxyphenyl on the side chain was shown to produce a sustained β-agonist response in an isolated tissue, suggesting that it is a slow dissociating β-agonist (Voss et al. 1992). In comparison to 11a, the selective β_2-agonist salmeterol has been shown to produce sustained activation of the β_2AR which may involve anchoring of the molecule's side chain to a receptor exosite. However, unlike 11a, salmeterol-mediated responses are attenuated by antagonists but can be reestablished after antagonist washout (Jack 1991; Coleman et al. 1996). Finally, it should be pointed out that although the simplest explanation of the present results is that 11a slowly dissociates from the β_2AR, other interpretations may be possible. For example, the data do not completely rule out 11a binding to and activating the β_2AR followed by dissociation leaving the receptor in a sustained activated conformation. However, this interpretation would require a reduced antagonist affinity for the activated receptor conformation because pretreatment of membranes with 11a reduced the B_{max} for [^{125}I]CYP binding. Additional studies will be necessary to investigate if a sustained activated receptor conformation after 11a dissociation is possible.

In summary, the present data indicate that the 8-hydroxycarbostyril nucleus and the positioning of the side chain geminal methyls and phenyl group are important agonist structural features leading to high affinity binding to, and slow dissociation from, the β_2AR. Further refinement of these structure-affinity relationships in conjunction with molecular studies on their specific interactions within the β_2AR may lead to the development of additional agonists which have a long duration of action based upon slow dissociation.

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