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Irreversible binding of a carbostyril-based agonist and antagonist to the β-adrenoceptor in DDT_1 MF-2 cells and rat aorta

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1 The chemoreactive ligands 5(2-(((1′-(4′-isothiocyanatophenylamino)thiocarbonyl)-amino)-2-methylpropyl)amino-2-hydroxypropoxy)-3,4-dihydrocarbostyril (DCITC) and 8-hydroxy-5(2-(((1′-(4′-isothiocyanatophenylamino)thiocarbonyl)amino)-2-methylprop-2-yl)amino-1-hydroxyethyl)-carbostyril (HCITC) were synthesized and shown to be potent irreversible antagonist and agonist ligands, respectively, for the β-adrenoceptor in DDT_1 MF-2 (DDT) cells and the rat isolated aorta.

2 In DDT cell membranes DCITC and HCITC inhibited (−)[^{125}I]-iodocyanopindolol (CYP) binding to the β-adrenoceptor with IC_{50} values of 1.1 and 18 nM, respectively. (−)-Isoprenaline inhibited [^{125}I]-CYP binding with an IC_{50} of 355 nM. Pretreatment of membranes with either chemoreactive ligand produced a time- and concentration-dependent decrease in the β-adrenoceptor content, indicating irreversible receptor binding. DCITC at concentrations up to 10 μM did not stimulate cyclic AMP accumulation in DDT cells nor did it amplify forskolin-stimulated cyclic AMP accumulation.

3 In the rat isolated aorta, DCITC (0.1 μM) did not affect either the phenylephrine-mediated tissue contraction or the acetylcholine-mediated relaxation. DCITC attenuated the maximal (−)-isoprenaline-mediated relaxation of a phenylephrine contracted aorta in a concentration-dependent manner and shifted the dose-response curves for (−)-isoprenaline to the right. The DCITC-induced decrease in maximal response was not reversed by extensive tissue washing. By use of the operational model of agonism, the calculated dissociation constant for (−)-isoprenaline ws 286 nM and the estimated receptor reserve for this agonist was 23% at the maximal response.

4 HCITC and (−)-isoprenaline stimulated cyclic AMP accumulation in DDT cells with pD_2 values (negative logarithm to base 10 of EC_{50}) of 7.95 and 7.97, respectively, and both mediated the same maximal stimulation. In the rat isolated aorta, HCITC produced a concentration-dependent relaxation of the tissue with a pD_2 value of 6.62, whereas the pD_2 for (−)-isoprenaline was 7.03. However, HCITC produced a greater maximal relaxation of the tissue than (−)-isoprenaline. The HCITC-mediated stimulation of cyclic AMP accumulation and relaxation of the isolated tissue were blocked when the β-antagonist propranolol was added concurrently. In contrast, once the HCITC-mediated responses were established, the addition of propranolol did not result in any attenuation indicating that HCITC is an irreversible β-agonist.

Keywords: β-Adrenoceptor; DDT_1 MF-2 cells; isolated aorta; chemoreactive ligands; (−)-isoprenaline; cyclic AMP; propranolol; 5(2-(((1′-(4′-isothiocyanatophenylamino)-thiocarbonyl)amino)-2-methylpropyl)amino-2-hydroxypropoxy)-3,4-dihydrocarbostyril (DCITC); 8-hydroxy-5(2-(((1′-(4′-isothiocyanatophenylamino)thiocarbonyl)amino)-2-methylprop-2-yl)amino-1-hydroxyethyl)-carbostyril (HCITC)

Introduction

Chemoreactive ligands are useful probes to study the structure and function of receptors. These ligands are usually composed of a pharmacophore based upon known agonists or antagonists, which provides affinity and selectivity for the receptor and a chemoreactive moiety through which covalent attachment to the receptor occurs preventing ligand dissociation. Antagonist based chemoreactive ligands have been used to determine agonist dissociation constants (K_A) and receptor reserve (Furchgott, 1996; Dennis *et al*., 1992), basal receptor turnover (Mahan *et al*., 1987), mapping of the antagonist binding site (Dohlman *et al*., 1988; Curtis *et al*., 1989) and to discriminate receptor subtypes (Han *et al*., 1987; Salles *et al*., 1993). Although fewer agonist based irreversible ligands have been described, they have additional potential in studies on the mechanism of receptor activation, desensitization and defining the agonist binding site in the receptor (Baker & Deyrup, 1994).

A number of chemoreactive ligands for the β-adrenoceptor have been synthesized and shown to act as irreversible ligands including those based upon the antagonist pharmacophores propranolol (NHNP-NBE, Atlas & Levitzki, 1976), alprenolol (BAAM, Pitha *et al*., 1980; Baker & Pitha, 1982) and a benzofuranyl derivative (Ro03-7894, Nicholson & Broadley, 1978; Rankin & Broadley, 1982). However, in subsequent studies irreversible inactivation of the β-adrenoceptor by NHNP-NBE and Ro03-7894 has been questioned (Krstew *et al*., 1984; Minneman & Mowry, 1986). Furthermore, in some but not all isolated tissue studies, Ro03-7894 and BAAM appeared to produce nonspecific tissue depression either in the absence or presence of irreversible receptor blockade, limiting their usefulness in these studies (Krstew *et al*., 1984; Posner *et al*., 1984; May *et al*., 1985; Ng & Malta, 1989; Doggrell, 1990). This nonspecific effect may be related to the relatively high concentration of chemoreactive ligand required for receptor blockade which also allows the reactive

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group to modify covalently nonreceptor tissue components. To overcome this problem, chemoreactive ligands with a high affinity pharmacophore should produce irreversible receptor binding at low ligand concentrations with minimal nonspecific reactions. Several chemoreactive ligands based on the high affinity pharmacophores pindolol (Br-AAM-pindolol) and cyanopindolol (BIM) have been shown to bind irreversibly to the β-adrenoceptor, with little or no nonspecific effects in depressing cell or isolated tissue responses (Homburger *et al*., 1985; Kusiak & Pitha, 1987; Jasper *et al*., 1988; Molenaar *et al*., 1988). In terms of chemoreactive agonists for the β-adrenoceptor, BAAM, BIM and a bromoacetylaminomenthyl derivative of nonadrenaline (BAAN) have been shown to have varying degrees of partial agonist activity (Baker *et al*., 1985; Jasper *et al*., 1991). In contrast, a carbostyril-based chemo-reactive ligand (carbo-Br) was shown to be an irreversible full β-agonist for the stimulation of adenylyl cyclase activity in an isolated membrane preparation (Standifer *et al*., 1989). The effects of this compound on β-adrenoceptor function in cultured cells and isolated tissues have not been described. Nonetheless, continual refinement in the chemical and pharmacological properties of antagonist and agonist based chemoreactive ligands for the β-adrenoceptor will enhance their usefulness in receptor studies.

In this study, the synthesis and pharmacological characterization of the β-antagonist 5(2-(((1′-(4′-isothiocyanatophenylamino)thiocarbonyl)amino) - 2 - methylpropyl) amino-2-hydroxypropoxy)-3,4-dihydrocarbostyril (DCITC) and the β-agonist 8-hydroxy-5(2-(((1′-(4′-isothiocyanatophenylamino)thiocarbonyl)amino)-2-methylprop-2-yl)amino-1-hydroxyethyl)-carbostyril (HCITC) are presented. The pharmacophore for these chemoreactive ligands is based on a carbostyril nucleus for which previous derivatives have been shown to be potent agonists and antagonists for the β-adrenoceptor (Nakagawa *et al*., 1974; Yoshizaki *et al*., 1976). In the synthesis of DCITC and HCITC, the chemoreactive moiety selected for incorporation into the pharmacophore is an isothiocyanate which can readily react with nucleophilic amino acids (Baker & Deyrup, 1994). This reactive moiety has been used in chemoreactive ligands for several other receptors (Jacobson *et al*., 1989; Kim *et al*., 1989; Newman *et al*., 1990) but has not been employed previously in irreversible ligands for the β-adrenoceptor. By use of DDT_1 MF-2 (DDT) cells and the rat isolated aorta preparation, DCITC was shown to be a potent, irreversible antagonist for the β-adrenoceptor and to be a suitable pharmacological probe for determining the K_A for agonists and the receptor reserve. On the other hand, HCITC was shown to be a potent β-agonist that produces sustained, antagonist-insensitive responses in DDT cells and the isolated tissue.

Methods

Analytical procedures

^1H and ^{13}C nuclear magnetic resonance (n.m.r.) spectra were measured on QE 300 MHz instrument with TMS as an internal standard in deuterated solvents as indicated. High resolution mass spectra (h.r.m.s.) were obtained by means of FAB ionization mode with CH_5^+. Precoated silica gel sheets (60F-254, 0.2 mm, EM-Reagents) were used for thin layer chromatography (t.l.c.). Preparative layer plates were used for microscale purification (silica gel GF, 20 × 20, 2 mm, Analtech). Silica gel (grade 60, 230–400 mesh, Merck Co.) was used for column chromatography.

Synthesis of DCITC

5-(2,3-Epoxy)propoxy-3,4-dihydrocarbostyril (0.43 g, 2 mmol) was reacted with monoprotected N-t-butoxycarbonyl-2-amino-2-methyl propylamine (0.565 g, 3 mmol) in boiling n-butanol (15 ml) for 6 h. The solvent was removed under vacuum and the reaction mixture purified by column chromatography on silical gel with methylene chloride methanol (98 : 2, 95 : 5, 90 : 10) to elute the product. The yield of purified material was 0.408 g, (50%). ^1H n.m.r. (DMSO): 1.05 (6H, s), 1.41 (9H, s), 2.45–2.55 (2H, t), 2.70–285 (2H, m), 2.95 (3H, t), 3.05 (2H, s), 4.00 (2H, s), 6.50 (1H, d), 6.65 (1H, d), 7.10 (1H, t). ^{13}C n.m.r. (DMSO): 23.63, 27.37, 29.59, 44.33, 53.46, 69.03, 70.66, 78.69, 106.32, 106.41, 108.36, 111.86, 127.60, 138.49, 155.92, 157.31, 172.37. h.r.m.s. 408.2497 (M + H). $C_{21}H_{34}N_3O_5$ requires 408.2498.

The product was deprotected by use of HCl saturated solution in 1,4-dioxane at room temperature overnight. The corresponding hydrochloride was very hygroscopic and used without further purification. A small amount of the hydrochloride (20 mg) was neutralized with sodium bicarbonate and analysed by mass spectrometry. H.r.m.s. 308.1994 (M + H). $C_{16}H_{26}N_3O_3$ requires 308.1974.

To 98 mg (0.285 mmol) of 5-[3-(2-amino-2-methylpropylamino)-2-hydroxypropoxy]-3,4-dihydrocarbostyril hydrochloride suspended in 1 ml of dry DMF triethylamine (0.079 ml, 0.285 mmol) was added, followed by an excess of 1,4-phenyldiisothiocyanate (238 mg, 1.24 mmol). This mixture was stirred at room temperature for 5 h. The solvent was removed under vacuum, and the crude material purified on preparative t.l.c. with silica gel and 15% methanol in methylene chloride as eluent. The stationary phase was extracted with the same solvent mixture yielding 54 mg (38%) of the pure product. ^1H n.m.r. (DMSO): 1.15 (6H, two s), 2.45–2.52 (2H, m), 2.65–2.90 (4H, m), 3.53 (1H, broad signal), 3.90–4.00 (3H, m), 5.50–5.55 (1H, m), 6.45 (1H, t), 6.55–6.62 (1H, m), 7.00–7.10 (1H, m), 7.15–7.35 (3H, m), 7.45–7.50 (1H, m). h.r.m.s. 500.1798 (M + H). $C_{24}H_{30}N_5O_3S_2$ requires 500.1790.

Synthesis of HCITC

8-(Benzyloxy)-5-oxiranyl-2(1H)-quinolinone (prepared by use of a modification of the procedure described by Milecki *et al*. (1987) (1.10 g, 3.82 mmol) and N-t-butoxycarbonyl-2-amino-2-methylpropylamine (1.2 g, 4.80 mmol) were suspended in n-butanol (6 ml) and heated for 4 h. The solvent was removed under vacuum, the residue dissolved in methylene chloride (30 ml) and extracted with HCl solution (15 ml, 1%). The organic layer was washed with water (15 ml), dried ($MgSO_4$) and the solvent removed. The residue was then chromatographed on a silica gel column with methylene chloride, followed by methylene chloride/methanol mixture from 2–5%. Yield was 0.956 g (54%). For $C_{27}H_{35}N_3O_5$ found: C 67.11, H 7.39, N 8.59. Required: C 67.34, H 7.33, N 8.72. ^1H n.m.r. (DMSO): 1.26 (6H, s) 1.38 (9H, s), 2.90–3.32 (4H, m), 3.44 (1H, broad signal), 5.35 (2H, s), 5.52 (1H, d), 6.58 (1H, d), 7.18–742 (5H, m), 7.60 (2H, d), 8.41 (1H, d), 10.85 (1H, broad signal). ^{13}C.m.r. (DMSO): 21.69, 21.79, 28.58, 46.10, 48.62, 59.77, 65.74, 70.12, 78.72, 112.58, 116.96, 119.98, 122.85, 128.29, 128.75, 129.81, 130.84, 136.87, 137.04, 144.16, 156.58, 161.31.

The protected aminoalcohol (2.6 g, 5.4 mmol) was dissolved in methanol (30 ml) and ammonium formate (500 mg), followed by palladium on charcoal (10%, 100 mg) was added at once under nitrogen. The reaction was refluxed and monitored by t.l.c. After no more starting material was detected the mixture was cooled to room temperature, filtered through fine filter and the filter concentrated under vacuum. The residue was purified by column chromatography on silica gel and 10% methanol in methylene chloride as eluent. Yield of the debenzylated product was 1.5 g (71%). For $C_{20}H_{29}N_3O_5$ found: C 60.96, H 7.80, N 10.65. Required: C 61.30, H 7.47, N 10.43. ^{1}H n.m.r. (DMSO): 0.916 (6H, s), 1.384 (9H, s), 2.624 (2H, d), 2.851 (2H, d), 4.900 (1H, t), 6.489 (1H, d), 6.917 (1H, d), 7.087 (1H, d), 8.182 (1H, d).

To remove the t-butoxycarbonyl group the protected aminoalcohol (1.8 g, 4.6 mmol) was suspended in dioxane solution of hydrogen chloride (15 ml, 4 M) and the mixture was stirred overnight. The solvent was removed under vacuum and the product, a white solid was dried over phosphorous pentoxide. Yield was 1.34 g (89%). ^{1}H n.m.r. (D_2O): 1.5 (6H, d), 3.28–3.41 (2H, m), 3.60–3.64 (2H, m), 5.30–5.35 (1H, m), 6.40 (1H, d), 6.75 (1H, d), 7.12 (1H, d), 8.05 (1H, d). ^{13}C n.m.r. (D_2O + dioxane): 20.88, 21.08, 45.08, 47.42, 58.23, 65.26, 114.65, 117.90, 119.21, 121.69, 121.73, 126.72, 138.09, 143.38, 162.35. H.r.m.s. 292.1590 (M + H). $C_{15}H_{22}N_3O_3$ requires 292.1661.

8-Hydroxy-5-[2-(2-amino-2-methylpropylamino)-1-hydroxyethyl]carbostyril hydrochloride (0.312 g, 0.952 mmol) was dissolved in 5 ml of dry DMF, then triethylamine (0.193 g, 0.266 ml, 1.9 mmol) was added followed by 1,4-phenyldiisothiocyanate (1 g, 5 mmol). The mixture was stirred at room temperature for 4 h and then DMF was removed under reduced pressure. The residue was purified on preparative t.l.c. plates (silica gel) by use of 10% methanol in methylene chloride as developing mixture. The silica gel was then scraped and extracted with the same mixture of solvents. Yield of the isothiocyanate was 0.21 g (47%). H.r.m.s. 484.1483 (M + H). $C_{23}H_{26}N_5O_3S_2$ requires 484.1477. ^{1}H n.m.r. (MeOD): 1.85 (6H, s), 3.55–3.70 (2H, m), 4.25–4.31 (2H, broad signal), 5.70–5.75 (1H, m), 7.27 (1H, d), 7.65 (1H, d), 7.85–7.95 (3H, m), 8.17 (2H, m), 8.90–9.02 (1H, m).

Drug preparations

Stock solutions (10 mM) of synthesized compounds were made in DMSO and diluted with HBSS before use. The dilution of vehicle (DMSO) was a minimum of 100 and 1000 fold for the isolated tissue and cultured cell experiments, respectively, and had no effect on responses in either preparation. All other drugs were made fresh at the time of use.

Cell culture

DDT cells were grown as monolayers in 57 cm^2 petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal bovine serum, 2.75 μg ml^{-1} amphotericin B, 100 u ml^{-1} penicillin G, and 0.1 mg ml^{-1} streptomycin sulphate in a humidified atmosphere of 95% air and 5% CO_2. Cells were routinely subcultured from stocks once or twice a week, with 1 mM EDTA in Hank's balanced salt solution (HBSS) without divalent cations to detach the cells and HBSS to disperse them. The cells were initially seeded at 1.2×10^5 cells/plate and experiments were performed 4 days later at 50–60% confluence (4×10^6 cells/plate). Cell viability was determined by exclusion of trypan blue.

Membrane preparations and pretreatments

Cell monolayers were washed twice with HBSS (2×10 ml), scraped free of the plate with the aid of a cell scraper 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 4 for 10 s. The suspension was then centrifuged at $15{,}000 \times g$ for 10 min. The pellet was resuspended in 50 mM Tris-HCl buffer pH 7.4 by vortexing and centrifuged as before. The final pellet was resuspended in 50 mM Tris-HCl buffer pH 7.4 containing 5 mM $MgCl_2$ for assays. The protein content was determined by the method of Bradford (1976), with bovine serum albumin as the standard.

In pretreatment experiments, membranes (2 mg protein ml^{-1}) were incubated in 50 mM Tris-HCl at pH 7.4 containing 5 mM $MgCl_2$, 100 μM 5′-guanylylimidodiphosphate (Gpp(NH)p) and without or with varying concentrations of DCITC or HCITC for 30 min at 36°C. At the end of the incubation, the suspensions were diluted to 25 ml with ice-cold incubation buffer and centrifuged at $48{,}000 \times g$ for 10 min. The pellet was then washed a further 9 or 5 times for membranes treated with DCITC or HCITC, respectively, by resuspension in 25 ml of buffer and centrifugation. The final membrane pellets were resuspended in 1 ml of buffer for assays.

Receptor binding assay

β-Adrenoceptors in cell membranes were determined by specific (−)[^{125}I]-iodocyanopindolol (CYP) binding (Standifer *et al*., 1989). Briefly, membrane protein (15 μg) was incubated in a total volume of 0.25 ml containing 50 mM Tris-HCl buffer at pH 7.4, 5 mM $MgCl_2$, [^{125}I]-CYP (6–100 pM), with or without 3 μM (±)-alprenolol for 60 min at 36°C. At the end of incubation, the cell suspensions were diluted with 3 ml of ice-cold incubation buffer and the suspension filtered through glass fibre filters under vacuum by use of a Brandel cell harvester. The filters were rinsed with a further 6 ml of ice-cold buffer and the retained radioactivity was determined with a gamma counter. Specific [^{125}I]-CYP binding to the β-adrenoceptor was calculated as a difference between the total binding in the absence of (±)-alprenolol and the nonspecific binding determined in the presence of (±)-alprenolol. Nonspecific binding was similar if the (±)-alprenolol was replaced with 100 μM (−)-isoprenaline and specific binding was 90–95% of the total bound.

In some experiments, the ability of several compounds to inhibit specific [^{125}I]-CYP binding was determined. The assays were the same as above except the [^{125}I]-CYP concentration was 30 pM and 100 μM Gpp(NH)p was included.

Cyclic AMP accumulation

Monolayers of DDT cells were rinsed with HBSS (2×10 ml), detached into HBSS (5 ml) using a cell lifter and the resulting suspension was centrifuged at $5000 \times g$ for 5 min. Aliquots of the cell suspension (0.5 mg protein) were then incubated in HBSS (500 μl total volume) for 5 min at 37°C, before addition of rolipram to final concentration of 50 μM and β-adrenoceptor ligands as indicated in text. The resulting suspensions were incubated for a further 10 min, placed in a boiling bath for 5 min, cooled to room temperature and then centrifuged at $13{,}000 \times g$ for 2 min. The adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) content of the supernatant was determined by a modification of a radioimmunoassay (Harper & Brooker, 1975). Briefly, an aliquot of the supernatant (50 μl) was added to 50 μl HBSS containing adenosine 3′,5′-cyclic

phosphoric acid 2′-O-succinyl[^{125}I]-iodotyrosine methyl ester ([^{125}I]-ScAMP, 10,000 c.p.m.). After the addition of anti-cyclic AMP antibody (50 μl of 1 : 2000 dilution with 0.1% BSA in water), the samples were incubated at 4°C for 18 h. At the end of incubation 70 μl of a 50% (v/v) hydroxyapatite suspension in water was added to each tube followed by incubation for 10 min at 4°C. The antibody/radioligand complex adsorbed to the hydroxyapatite was retained on glass fibre filters during rapid filtration with the Brandel cell harvester. The filters were washed with 6 ml of ice-cold 10 mM Tris-HCl buffer at pH 7.0. Nonspecific binding of [^{125}I]-ScAMP was determined in parallel assays that contained unlabelled cyclic AMP (0.1 μM), and was subtracted from the total binding. The amount of cyclic AMP was calculated from a standard curve constructed with known amounts of unlabelled cyclic AMP.

Tension measurements

Male rats were killed and the aorta was rapidly excised and placed in cold, oxygenated (95% O_2/5% CO_2) Krebs-Henseleit Buffer (KHB), containing in mM: NaCl 120, $NaHCO_3$ 20, KCl 4.2, KH_2PO_4 1.2, $MgCl_2$ 0.5, $CaCl_2$ 1.8 and glucose 11 at pH 7.4. A 3–4 cm segment of the aorta was dissected free and cleaned of fat and adventitia. Ring segments (3–5 mm long) were mounted onto two triangular tungsten wires and placed into a 10 ml isolated organ chamber. The bottom triangle was mounted to a stable hook while the top triangle was attached to a Gould strain gage. The bath was maintained at 37°C and a resting force of 1.5 g was applied to the aortic rings. Vessel segments were initially equilibrated for 2 h with alternating 5 min exposures to KCl (80 mM) and phenylephrine (1 μM). All concentration-response curves were performed cumulatively.

Data analysis

The IC_{50} values from radioligand binding assays and EC_{50} values from functional assays were determined from nonlinear regression analysis of the curves by use of the GraphPad Inplot programme (Graphpad Software, San Diego, CA). The dissociation constant (K_d) and maximal [^{125}I]-CYP binding was determined from nonlinear regression analysis (GraphPad Inplot). Statistical analysis of paired data was performed by use of Student's *t*-test.

The K_A and receptor reserve for (−)-isoprenaline in the isolated aorta was determined by simultaneously curve fitting the concentration-response curves using the operational model of agonism as described by Black and Leff (1983) with a multiple function nonlinear regression technique which used the Marquardt-Levenberg algorithm (SigmaPlot 4.0, SPSS Inc., Chicago, IL). The experimental data were fit to two forms of the operational model. Equations 1 and 2 predict the behaviour of nonhyperbolic (slope factor ≠1) and a rectangular hyperbolic (slope factor = 1) shaped concentration-response curves, respectively,

$$E = \frac{E_{max}\tau^n[A]^n}{([A]^n + K_A) + \tau^n[A]^n} \quad (1)$$

$$E = \frac{E_{max}\tau[A]}{= K_A + (1+\tau)[A]} \quad (2)$$

where E is the observed response, E_{max} is the theoretical maximal response. A is agonist concentration, τ is the operational efficacy of the agonist, n is the slope factor and K_A is the agonist equilibrium dissociation constant. The family of concentration-response curves were fitted assuming a common value of E_{max}, K_A and n (Equation 1). Only τ, which at submaximal levels of response decreases proportionally with the remaining fraction of non-inactivated receptors ($\tau = \tau' q$), was allowed to vary between individual concentration-response relations during the curve fitting process. Unless equation 1 gave a statistically improved fit ($P < 0.05$) compared to equation 2, the latter was used in the analysis.

Materials

DDT cells, a smooth muscle cell line derived from lieomyosarcoma of the ductus deferens of the syrian hamster, was obtained from American Type Culture Collection (Rockville, MD). (−)[^{125}I]-CYP (2000–2200 Ci mmol^{-1}) was purchased from Amersham Corp (Arlington Heights, IL). [^{125}I]-ScAMP was prepared by the method described by (Patel & Linden, 1988) and was generously provided by Dr John Shryrock from the Department of Medicine at the University of Florida. All other chemicals, including cyclic AMP, Gpp(NH)p, (−)-isoprenaline, (±)-alprenolol, 2′-O-monosuccinyladenosine 3′,5′-cyclic monophosphate tyrosyl methyl ester and hydroxyapatite were purchased from Sigma Chemical Co. (St. Louis, MO). Glass fibre filters were from Schleicher and Schuell (Keene, NH).

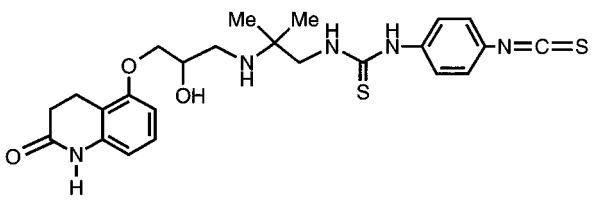
Results

Synthesis of DCITC and HCITC

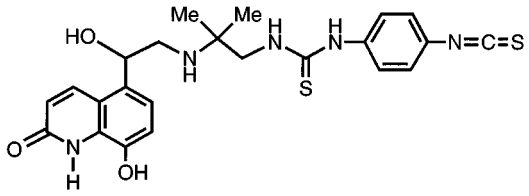
The multistep synthesis of DCITC was carried out by use of previously published procedures (Tamura *et al*., 1970; Dubas-Sluyter *et al*., 1972; Shono *et al*., 1981). 5-(2,3-Epoxy)propoxy-3,4-dihydrocarbostyril, prepared in a good yield, was reacted with N-t-butoxycarbonyl-2-amino-2-methylpropylamine (Wang *et al*., 1986) to yield the corresponding adduct, which was subsequently cleaved with hydrogen chloride etheral solution to give the amine hydrochloride. The amine hydrochloride was then reacted with phenyl 1,4-diisothiocyanate to give the chemoreactive antagonist. Similarly, HCITC was synthesized starting with the corresponding 8-(benzyloxy)-5-oxiranylcarbostyril (Milecki *et al*., 1987). This epoxide was treated with N-t-butoxycarbonyl-2-amino-2-methylpropylamine to give the protected adduct which was hydrogenated (to remove benzyl group) and reacted with hydrogen chloride to deprotect the amino group. The amine hydrochloride was coupled with phenyl 1,4-diisothiocyanate to render the corresponding chemoreactive agonist. The structures of the two chemoreactive compounds are shown in Figure 1.

Effect of DCITC and HCITC on the β-adrenoceptor in DDT cells

The effect of DCITC, HCITC and the β-agonist (−)-isoprenaline on [^{125}I]-CYP binding to the β-adrenoceptor in DDT cell membranes is shown in Figure 2a. These competition assays were performed in the presence of 100 μM Gpp(NH)p to maintain the receptor in the agonist low affinity state (Lefkowitz *et al*., 1976; Kent *et al*., 1980). The concentration of (−)-isoprenaline that inhibited specific [^{125}I]-CYP binding by 50% (IC_{50}) was 355 ± 25 nM ($n = 5$). Both DCITC and HCITC were relatively potent as inhibiting [^{125}I]-CYP binding with IC_{50} values of 1.1 ± 0.5 and 18 ± 3 nM, respectively. The agonist effects of the three compounds were examined by the stimulation of cyclic AMP accumulation in DDT cells. As depicted in Figure 2b, (−)-isoprenaline and HCITC increased cyclic AMP accumulation in a concentration-dependent



DCITC



HCITC

Figure 1 Structure of DCITC and HCITC.

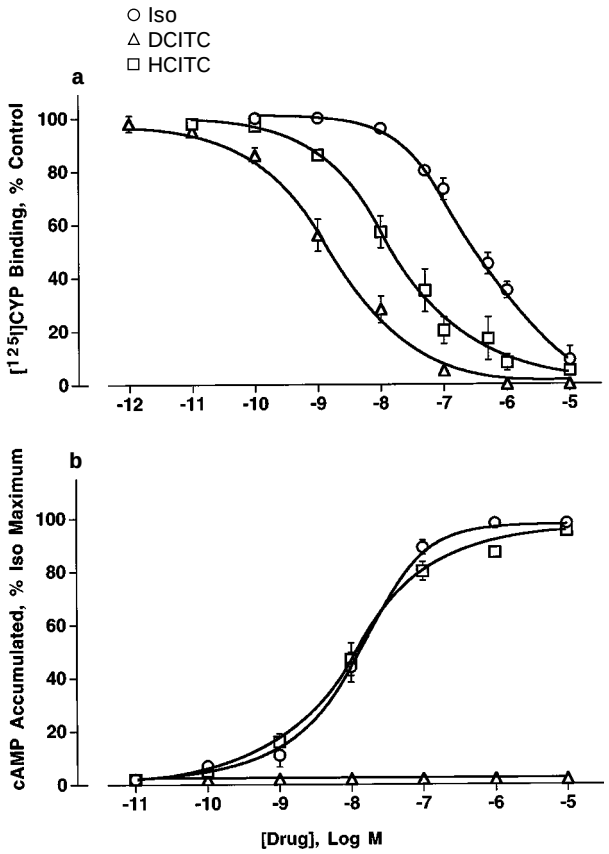


Figure 2 Concentration-dependent effect of (−)-isoprenaline (Iso), DCITC and HCITC to (a) inhibit [^{125}I]-CYP binding in DDT cell membranes and (b) stimulate cyclic AMP accumulation in DDT cells. (a) DDT cell membranes were incubated in the presence of 100 μM Gpp(NH)p, 30 pM [^{125}I]-CYP and the indicated concentration of ligand for 60 min at 36°C. The control [^{125}I]-CYP binding was 63 ± 3 fmol mg^{-1} protein. (b) DDT cells were incubated with 50 μM rolipram and the indicated concentration of ligand for 10 min at 37°C. The basal and maximal (−)-isoprenaline-stimulated cyclic AMP accumulation were 45 ± 2 and 515 ± 68 pmol mg^{-1} protein 10 - min^{-1}, respectively. Data from both series of experiments are the mean with vertical lines showing s.e.mean; $n=3-4$.

manner producing the same maximal stimulation above basal levels [(−)-isoprenaline, 11.2 ± 2 fold, and HCITC, 11.5 ± 1.5 fold, $n=6$]. The pD_2 values (negative logarithm to base 10 of the EC_{50}) was 7.97 ± 0.08 (mean ± s.e., $n=6$) for (−)-isoprenaline and 7.95 ± 0.04 (mean ± s.e., $n=6$) for HCITC. In contrast, DCITC did not increase cyclic AMP accumulation at concentrations up to 10 μM (Figure 2b). Jasper *et al.* (1990, 1991) showed that the weak partial agonist activity for several β-adrenoceptor ligands can be readily observed by amplification of forskolin-stimulated cyclic AMP accumulation in several cell lines. This experimental approach was used to test further for DCITC agonist activity. Forskolin (1 μM) and (−)-isoprenaline (0.1 μM) increased cyclic AMP accumulation in DDT cells 7.9 ± 0.6 ($n=3$) and 15.8 ± 0.3 ($n=3$) fold above basal levels, respectively. In the presence of both forskolin and (−)-isoprenaline, there was an increase in the stimulation fold to 28.9 ± 2.4 ($n=3$, $P<0.005$ vs forskolin alone). However, when DCITC (0.1 μM) was incubated with forskolin, there was no change in the fold stimulation of cyclic AMP accumulation (7.8 ± 1.0, $n=3$) as compared to forskolin alone.

To investigate further the interaction of HCITC and DCITC with the β-adrenoceptor, DDT cell membranes were incubated with these compounds in the presence of 100 μM Gpp(NH)p, followed by membrane washing and nonlinear regression analysis of specific [^{125}I]-CYP binding. As shown in Table 1, preincubation of DDT cell membranes with 0.5, 1 and 5 nM DCITC for 30 min at 36°C followed by 10 membrane wash cycles resulted in a 30, 47 and 75% decrease in [^{125}I]-CYP binding, respectively, with no change in the K_d value of the radioligand for the receptors remaining. HCITC also produced a concentration-dependent decrease of β-adrenoceptors. Incubation of DDT cell membranes for 30 min at 36°C with 5 nM HCITC followed by 6 membrane wash cycles reduced specific [^{125}I]-CYP binding by 43%. Incubation with 10 nM HCITC reduced specific binding by 60% and with 100 nM HCITC the reduction was 84%. For each concentration of HCITC used there was no change in the K_d value for [^{125}I]-CYP binding to the receptors remaining (Table 1). The DCITC and HCITC-induced loss of [^{125}I]-CYP binding to the β-adrenoceptor was time-dependent (Figure 3). Incubation of DDT cell membranes with 10 nM DCITC or 100 nM HCITC in the presence of 100 μM Gpp(NH)p reduced [^{125}I]-CYP binding by 54 and 30%

Table 1 Effect of DCITC and HCITC on β-adrenoceptors in DDT$_1$ MF-2 cell membranes

DCITC pretreatment	*[^{125}I]-CYP B_{max}* (fmol mg^{-1} protein)	K_d (nM)
Control	173 ± 11 (3)	26 ± 2
0.5 nM	126 ± 17 (3)*	27 ± 5
1.0 nM	96 ± 3 (3)**	28 ± 3
5.0 nM	47 ± 4 (3)***	28 ± 1
HCITC pretreatment		
Control	228 ± 7 (4)	43 ± 4
5 nM	134 ± 13 (3)***	40 ± 3
10 nM	91 ± 5 (4)***	42 ± 4
100 nM	30 ± 5 (3)***	34 ± 2

Membranes were incubated with 100 μM Gpp(NH)p and the indicated concentrations of DCITC or HCITC for 30 min at 36°C. At the end of the incubation, the membranes were washed (DCITC, 10 times; HCITC, 6 times) and the B_{max} and K_d for [^{125}I]-CYP binding to the β-adrenoceptor were determined. Data are the means ± s.e. Values in parentheses are the number of experiments. *$P<0.05$, **$P<0.01$, ***$P<0.0005$ compared to respective control.

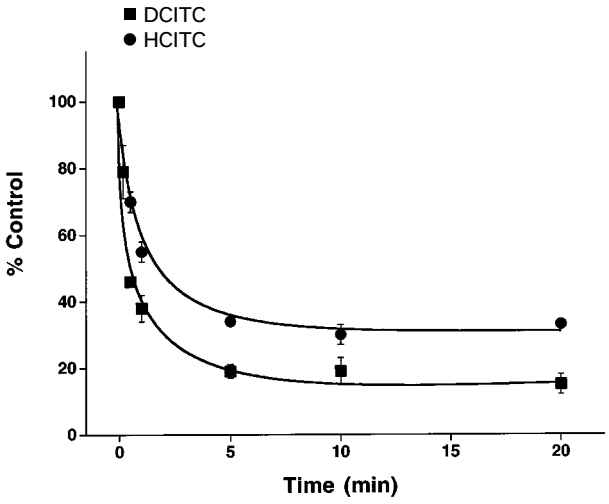


Figure 3 Time course of DCITC and HCITC-induced loss of specific [^{125}I]-CYP binding. DDT cell membranes were incubated with 10 nM DCITC or 100 nM HCITC in the presence of 100 μM Gpp(NH)p. At the times indicated, the membranes were washed and the specific binding of 100 pM [^{125}I]-CYP determined. Data are the mean with vertical lines showing s.e.mean; $n=3-4$. The control [^{125}I]-CYP binding at time 0 was 133 ± 12 fmol mg^{-1} protein.

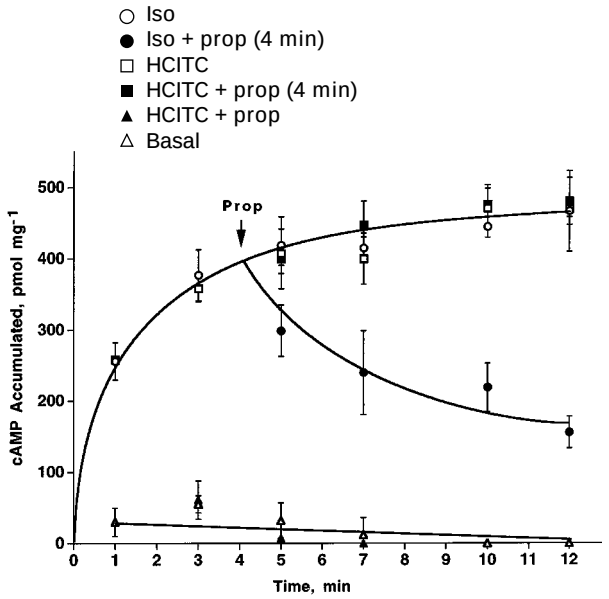


Figure 4 Time course of (−)-isoprenaline and HCITC mediated stimulation of cyclic AMP accumulation. DDT cells were incubated with 50 μM rolipram and 1 μM (−)-isoprenaline (Iso) or 1 μM HCITC and at the times indicated the cyclic AMP content was determined. Propranolol (Prop; 20 μM) was added after 4 min of incubation with (−)-isoprenaline (Iso + prop, 4 min) or HCITC (+prop, 4 min). HCITC and propranolol added together (HCITC + -prop) and basal cyclic AMP. Data are the mean with vertical lines showing s.d. of triplicate determinations and is representative of 3 experiments.

after 0.5 min and reached a maximal reduction of 81 and 70%, respectively, by 10 min of incubation.

The β-antagonist propranolol was used to investigate the reversibility of (−)-isoprenaline and HCITC-mediated stimulation of cyclic AMP accumulation in DDT cells. As shown in Figure 4, both (−)-isoprenaline (1 μM) and HCITC (1 μM) increased cyclic AMP accumulation in a similar time-dependent manner with 256 ± 26 (mean $\pm$ s.d.) pmol cyclic AMP formed after 1 min of incubation and this was increased to 467 ± 57 after 12 min. When an excess of propranolol (20 μM) was added after a 4 min incubation with (−)-isoprenaline alone, there was a subsequent time-dependent decrease in cyclic AMP accumulation to 33% of the maximal stimulated level at the end of the 12 min incubation period. This indicates that the (−)-isoprenaline-mediated increase in cyclic AMP accumulation, and the interaction of (−)-isoprenaline with the β-adrenoceptor is reversible. In contrast, when propranolol was added after a 4 min incubation with HCITC alone, the rate of cyclic AMP accumulation was the same as that observed in the absence of the antagonist. When propranolol was added concurrently with HCITC, the stimulation of cyclic AMP accumulation was blocked over the 12 min incubation (Figure 4). This demonstrated that once bound to the β-adrenoceptor HCITC produced an antagonist-insensitive response, consistent with this compound binding to the receptor in an irreversible manner.

Effect of DCITC on the rat isolated aorta

The effect of DCITC on (−)-isoprenaline-mediated relaxation of the rat isolated aorta contracted with 500 nM phenylephrine is shown in Figure 5a. In these experiments, the tissue was incubated with DCITC for 30 min before the effects of (−)-isoprenaline were determined. This preincubation period (30 min) was used because the maximal DCITC-induced receptor loss in DDT cells occured before this time (Figure 3). (−)-Isoprenaline alone produced a concentration-dependent relaxation of the smooth muscle preparation with an pD_2 of 7.02 ± 0.09 and a maximal relaxation of $71\pm4.4\%$ ($n=4$). After exposure of the tissue to 1 or 10 nM DCITC for 30 min, the (−)-isoprenaline pD_2s were 6.95 ± 0.08 ($n=5$) and 6.68 ± 0.06 ($n=6$, $P<0.005$ vs control) and the maximal relaxation was reduced to 61 ± 13 and $31.8\pm1.5\%$, respectively. Pretreatment of the tissue with 100 nM DCITC further attenuated the maximal (−)-isoprenaline-induced relaxation to $9.0\pm2.8\%$ ($n=3$) with a pD_2 of 6.39 ± 0.04 ($n=3$, $P<0.005$ vs control).

The reversibility of the DCITC effect on (−)-isoprenaline-induced relaxation was investigated by tissue washout experiments. As depicted in Figure 5b, the maximal (−)-isoprenaline-mediated relaxation of the control was $75.8\pm1.3\%$ ($n=5$) and this relaxation was decreased to $34.1\pm1.9\%$ ($n=5$) after pre-exposure of the tissue for 30 min to 10 nM DCITC. After a 1 h washout period no recovery of the maximal relaxation was observed ($38.8\pm0.6\%$, $n=5$).

To determine if DCITC produced non-β-adrenoceptor-mediated tissue effects, the response to phenylephrine and acetylcholine in the presence of DCITC was determined. Phenylephrine (500 nM) alone increased aortic contraction and this contraction was transiently attenuated after the addition of acetylcholine (1 μM). Neither the phenylephrine contraction nor the acetylcholine relaxation was affected in the presence of 0.1 μM DCITC (Figure 6).

K_A and receptor reserve for (−)-isoprenaline in rat isolated aorta

Compared to the hyperbolic form of the operational model of agonism, the more complex form (i.e., nonhyperbolic form) did not statistically improve the fit of the concentration-response data depicted in Figure 5a ($P=0.42$). By use of the hyperbolic form, the estimated K_A and operational efficacy (τ) of (−)-isoprenaline were 286 ± 92 nM and 1.63 ± 0.97, respectively. Pretreatment of the aortic tissue with 1, 10 or 100 nM

DCITC, not only cause a concentration-dependent reduction in the magnitude of the maximal relaxation (Figure 5a), but also in the fraction of non-inactivated receptors (q) to 0.71 ± 0.07, 0.22 ± 0.06 and 0.05 ± 0.018, respectively. A plot of q (%) against maximal relaxation at each concentration of DCITC pretreatment revealed an occupancy-response relationship that was slightly hyperbolic (Figure 7). The receptor reserve for the (−)-isoprenaline-mediated maximal relaxation, defined as 90% of the maximum, is 23%.

Effect of HCITC on the rat isolated aorta

In phenylephrine-precontracted aortae, (−)-isoprenaline and HCITC produced a concentration-dependent relaxation (Figure 8). The pD_2 values were 7.03 ± 0.03 ($n=6$) for (−)-isoprenaline and 6.62 ± 0.03 ($n=6$) for HCITC. The maximal relaxation for (−)-isoprenaline and HCITC was $62 \pm 0.3\%$ and $97 \pm 0.6\%$ ($P<0.05$), respectively. The relaxation produced by both agonists (1 μM) was blocked in the presence of 10 μM propranolol (data not shown). The reversibility of the agonist-induced relaxation was examined by the use of propranolol (Figure 9). (−)-Isoprenaline (1 μM) and HCITC (1 μM) produced a 40 and 80% relaxation, respectively, of the phenylephrine contracted aorta. After the subsequent addition of 20 μM propranolol, the (−)-isoprenaline relaxation was rapidly and completely reversed. In contrast, the addition of propranolol had no effect on the HCITC-mediated relaxation, demonstrating that this agonist produced an antagonist-insensitive relaxation.

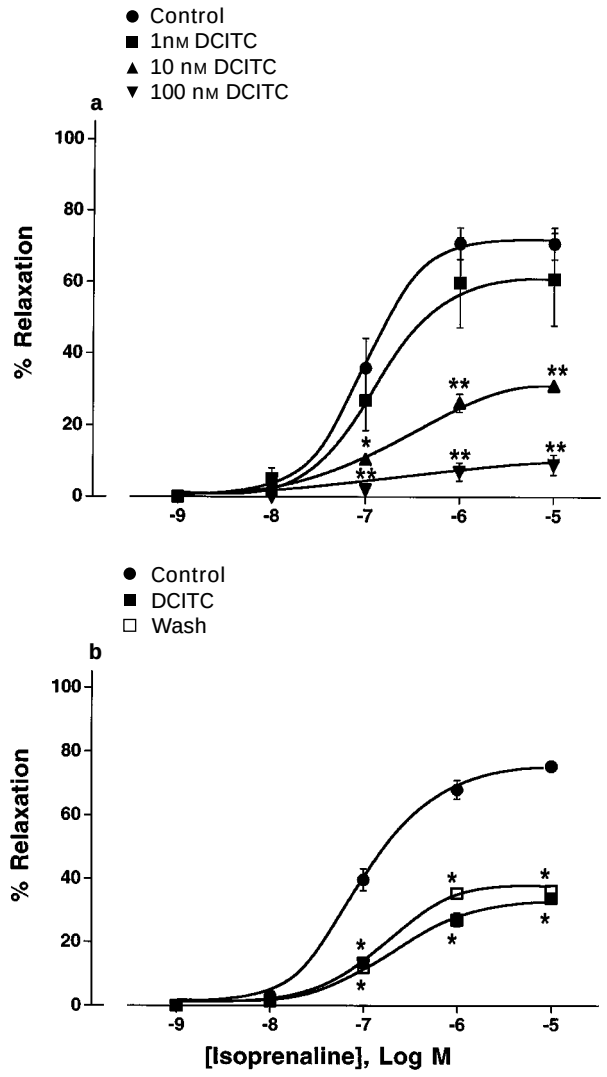


Figure 5 Effect of DCITC on (−)-isoprenaline-mediated relaxation of rat aorta. In (a): aortae were precontracted with phenylephrine (500 nM) and the tissue was exposed to solution in the absence (control) or presence of 1, 10 or 100 nM DCITC for 30 min. The concentration-response for relaxation by (−)-isoprenaline was then determined. Data are the means, $n=3-6$. $^*P<0.05$ and $^{**}P<0.01$ compared to the control (−)-isoprenaline values. In (b) aortae were contracted with 500 nM phenylephrine and the tissue was then incubated in the absence (control) or presence of 10 nM DCITC for 30 min followed by the concentration-response for (−)-isoprenaline. The tissue was then washed over a period of 60 min during which the tissue bath solution was exchanged with drug free solution three times every five min. At the end of the wash period, the concentration-response to (−)-isoprenaline was repeated (Wash). Data are the means, $n=5$. $^*P<0.01$ compared to control. In (a) and (b), vertical lines show s.e.mean.

Discussion

The results from the present study show that DCITC and HCITC are potent ligands acting as an antagonist and agonist, respectively for the β-adrenoceptor. Under the incubation

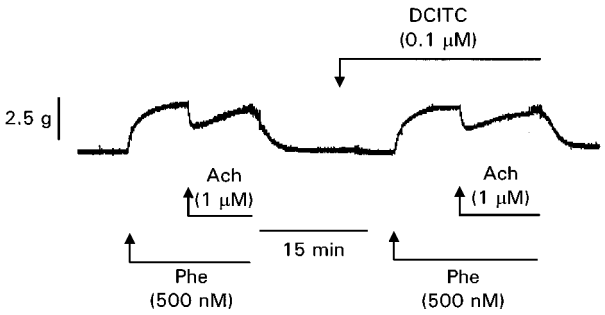


Figure 6 Phenylephrine contraction and acetylcholine relaxation of rat aorta in the presence of DCITC. Aortae were contracted with 500 mM phenylephrine and at the maximal contraction, acetylcholine (1 μM) was added. After washing to remove drugs the phenylephrine contraction and acetylcholine relaxation was repeated in the presence of 0.1 μM DCITC. Tracing is representative of 3 separate experiments.

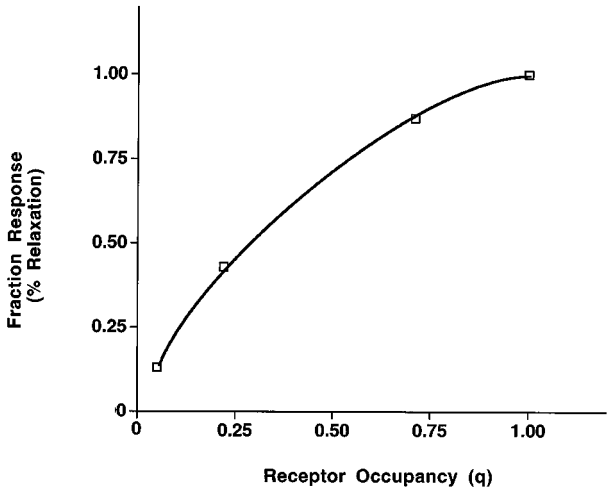


Figure 7 Occupancy-response relationship for (−)-isoprenaline-mediated relaxation of rat isolated aorta. Receptor occupancy (q) was calculated from (−)-isoprenaline dose-response curves (Figure 5a) after treatment with 1, 10, and 100 nM DCITC by use of the operational model.

conditions used in the radioligand binding assays (60 min, 36°C), these ligands inhibited [^{125}I]-CYP binding to the β-adrenoceptor in DDT cell membranes in the low nanomolar range. In the functional assays, the potency of HCITC to stimulate cyclic AMP accumulation in DDT cells and to relax the rat isolated aorta was in the low to mid nanomolar range. However, for several reasons, the potency of DCITC and HCITC may be underestimated. First, as discussed below, DCITC and HCITC are irreversible ligands and as such their apparent potency will increase as a function of incubation time. Second, it is well known that the (−)-enantiomer of β-agonists and antagonists are more potent than the (+)-enantiomers or their racemic mixtures (Patil *et al*., 1974). In the present study, both DCITC and HCITC were used as racemic mixtures and it is likely that their (−)-enantiomers will be more potent.

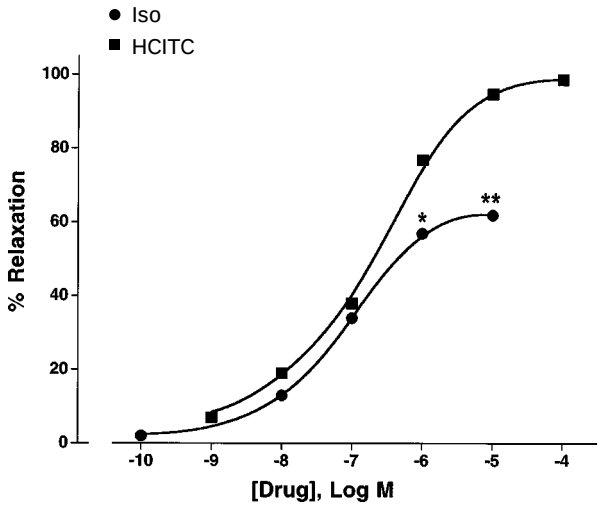


Figure 8 Relaxation effect of (−)-isoprenaline and HCITC on the rat aorta. Aortae were precontracted with 500 nM phenylephrine and the relaxation determined in the presence of the indicated concentrations of (−)-isoprenaline (Iso) or HCITC. Data are the mean and s.e., $n=6$. *$P<0.01$ and **$P<0.05$ compared to the (−)-isoprenaline values.

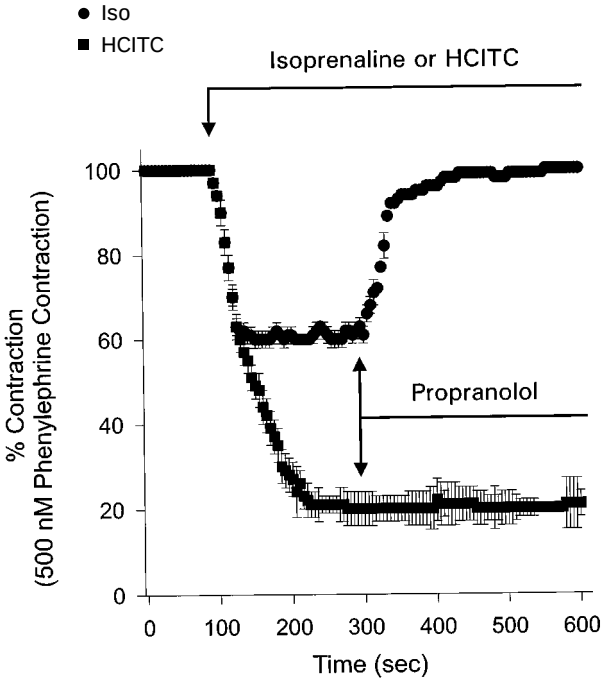


Figure 9 Effect of propranolol on (−)-isoprenaline and HCITC mediated relaxation in rat aorta. Aortae were contracted with 500 nM phenylephrine and then exposed to 1 μM (−)-isoprenaline (Iso) or 1 μM HCITC. After a stable relaxation was established, propranolol (20 μM) was added. Data are the means, $n=3$; vertical lines show s.e.mean.

DCITC alone did not increase cyclic AMP accumulation in DDT cells. Furthermore, DCITC unlike (−)-isoprenaline did not amplify forskolin-stimulated cyclic AMP accumulation in DDT cells, an experimental approach previously used to detect partial agonist activity of β-adrenoceptor ligands (Jasper *et al*., 1991). These observations are consistent with DCITC acting as a β-adrenoceptor antagonist without detectable partial agonist activity. In DDT cells, HCITC produced the same maximal stimulation of cyclic AMP accumulation as (−)-isoprenaline indicating that this chemoreactive ligand is a full β-agonist. However, in the isolated aorta, HCITC produced a greater maximal relaxation than (−)-isoprenaline. This difference in the maximal relaxation response for the two agonists may reflect a differential ability to activate the cellular transduction mechanisms coupled to the β-adrenoceptor in the smooth muscle cells of the isolated tissue (Kenakin, 1996).

A number of criteria, with radioligand binding assays, have been used to establish the irreversible binding of a chemoreactive ligand to a receptor. These include: (1) loss of receptor binding capacity as a function of chemoreactive ligand concentration and incubation time and (2) lack of receptor recovery with extensive washing to remove free ligand (Baker & Deyrup, 1994). In the present study, DCITC and HCITC reduced maximal [^{125}I]-CYP binding to the β-adrenoceptor in DDT cell membranes in a concentration-dependent manner, without a change in the K_d value for the radioligand to the receptors remaining. Furthermore, the DCITC and HCITC-induced reduction in receptor content was time-dependent and not reversed by extensive membrane washing. In addition, exposure of the isolated aorta to DCITC reduced in a concentration-dependent manner, the maximal (−)-isoprenaline-induced relaxation and this reduction was not reversed by extensive washing of the tissue. These findings with DDT cells and the isolated aorta are consistent with DCITC and HCITC binding to the β-adrenoceptor in an irreversible manner. By use of pharmacological criteria, the majority of β-adrenoceptors expressed in DDT cells and mediating relaxation of the rat aorta are the β_2 subtype (Norris *et al*., 1983; O'Donnell & Wanstall, 1984; Hadcock & Malbon, 1988). Thus, the present data indicate that DCITC and HCITC are irreversible ligands for the β_2-adrenoceptor. Further studies will be needed to determine if they bind in an irreversible manner to the other β-adrenoceptor subtypes. The previously described irreversible antagonists BAAM (Pitha *et al*., 1980), Br-AAM-pindolol (Homburger *et al*., 1985) and BIM (Kusiak & Pitha, 1987), and the irreversible agonist carbo-Br (Standifer *et al*., 1989), use an electrophilic haloacetamide as the reactive moiety that may form a covalent bond by reaction with nucleophilies in the receptor by a second order S_N2 process. In contrast, DCITC and HCITC contain an electrophilic isothiocyanate group as the reactive moiety, that can undergo an addition reaction when a receptor nucleophile attacks the electrophilic carbon of the ligand leading to the formation of a stable bond (Baker & Deyrup, 1994). DCITC and HCITC are the first β-adrenoceptor ligands which contain the isothiocyanate moiety and the data indicate that this reactive group is useful in the development of chemoreactive irreversible ligands for this receptor.

Several lines of evidence indicated that DCITC produced little if any nonspecific depression of responses in DDT cells or the rat isolated aorta at concentrations that irreversibly blocked the β-adrenoceptor. Forskolin, which activates adenylyl cyclase through a nonreceptor mechanism (Darfler *et al*., 1982), stimulated cyclic AMP accumulation in DDT cells. This increase was not affected by DCITC at a concentration (100 nM) greater than that required for receptor inactivation (0.5–10 nM). In the isolated aorta preparation, DCITC did not affect the basal tone or the contraction mediated by the α-adrenoceptor agonist phenylephrine. Furthermore, DCITC did not affect aortic relaxation mediated by acetylcholine-induced nitric oxide production. However, because of the high reactivity of the isothiocyanate moiety, it is possible that DCITC will cause some nonspecific tissue depression at high concentrations by reaction with non-receptor cellular or tissue components. Nonspecific tissue depression has been shown for the irreversible β-adrenoceptor antagonist BAAM (Posner *et al*., 1984; Ng & Malta, 1989). Nonetheless, the observation that DCITC can irreversibly block the β-adrenoceptor at concentrations that do not appear to cause any nonspecific effects, indicates that this compound will be useful on this receptor in a variety of biological systems.

By use of the operational model of agonism developed by Black and Leff (1983), the calculated K_A for the (−)-isoprenaline-mediated relaxation of aortic smooth muscle was 286 ± 92 nM. This value is similar to the experimentally derived EC_{50} values for this agonist obtained after treatment of the isolated tissue with 10 (218 ± 29 nM) or 100 (409 ± 33 nM) DCITC. Furthermore, by use of the Furchgott (1966) method, May *et al*. (1985) obtained a K_A value of 281–330 nM for (−)-isoprenaline to decrease the twitch response of the rat isolated vas deferens, a β_2-adrenoceptor response. Their K_A range is in excellent agreement with the K_A for (−)-isoprenaline determined in the present study. Based upon the q values (fraction of non-inactivated receptors) derived from the operational model, the β-adrenoceptor occupancy-response relationship for aortic muscle relaxation was slightly hyperbolic with a 23% receptor reserve for (−)-isoprenaline at the maximal response. This receptor reserve at the maximal response is similar to that obtained (19%) for the selective β_2-agonist procaterol mediating a relaxation of the rat isolated aorta (Doggrell, 1989), and suggests a tight coupling between the receptor and its intracellular processes that lead to muscle relaxation. However, the receptor reserve at 50% of the maximal response was about 20% for (−)-isoprenaline (Figure 7) and 45% for procaterol (Doggrell, 1989). This difference in receptor reserve for the two agonists at a submaximal response probably reflects differences in their intrinsic efficacies (Kenakin, 1993).

The results with DDT cells and the rat isolated aorta preparation demonstrated that HCITC produced sustained, antagonist-insensitive β-adrenoceptor responses. This was shown by the ability of propranolol to reverse the established HCITC-mediated increase in the rate of cyclic AMP accumulation in DDT cells or the HCITC-mediated relaxation of the aorta. These antagonist-insensitive responses were due to activation of the β-adrenoceptor because they were prevented when propranolol was added concurrently with HCITC. As expected for a reversible ligand, the (−)-isoprenaline-mediated increase in cyclic AMP accumulation and relaxation of the smooth muscle preparation was rapidly reversed by the addition of propranolol. These observations in conjuction with the radioligand binding data demonstrate that HCITC is an irreversible agonist for the β-adrenoceptor. In comparison, Baker *et al*. (1985) showed that a bromoacetylaminomenthyl derivative of noradrenaline (BAAN) could bind to the β-adrenoceptor in an irreversible manner. However, this compound was a weak partial agonist, chemically unstable, and although it initially stimulated adenylyl cyclase activity in membrane preparations, after irreversible binding it appeared to act as an antagonist. The reason for the difference between HCITC acting as an irreversible agonist and the transient agonist effects of BAAN is not clear. However, the difference may be related to BAAN alkylating a critical amino acid residue required for receptor activation, whereas HCITC may acylate a noncritical residue in the receptor. Chemoreactive agonists that produce sustained or transient responses for the muscarinic receptor have also been described (Ringdahl *et al*., 1984; Bolden & Baker, 1990; Crocker & Russell, 1990). A carbostyril-based β-agonist with a haloacetamide as the reactive moiety (carbo-Br) has been shown to bind irreversibly to the β-adrenoceptor and produce antagonist-insensitive activation of adenylyl cyclase activity in reticulocyte membranes (Standifer *et al*., 1989). Although HCITC and carbo-Br both appear to be potent irreversible ligands in isolated membrane preparations, the latter compound has not been studied in cultured cells or isolated tissue preparations.

In summary, the data from the present study show that DCITC and HCITC are potent, irreversible ligands for the β-adrenoceptor in DDT cells and the rat isolated aorta. DCITC is an irreversible antagonist which produces no significant nonspecific cell or tissue depressant effects at concentrations that inactivate the receptor. This chemoreactive ligand appears useful for determining the K_A and receptor reserve of agonists. HCITC, on the other hand, is an irreversible full β-agonist. The characteristics of these compounds suggest that they are potentially useful ligands in further studies on the structure and function of the β-adrenoceptor.

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