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Interaction of the antiarrhythmic agents SR 33589 and amiodarone with the β-adrenoceptor and adenylate cyclase in rat heart

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1 The effects of SR 33589 and amiodarone on the cardiac β-adrenoceptor were studied *in vitro* and after chronic treatment by means of [^{125}I]-(-)-iodocyanopindolol ([^{125}I]-(-)-CYP) binding and measurement of adenylate cyclase activity.

2 Binding of [^{125}I]-(-)-CYP was inhibited in a dose-dependent manner by SR 33589 ($IC_{50} = 1.8 \pm 0.4$ μM, $n_H = 0.93 \pm 0.06$) and amiodarone ($IC_{50} = 8.7 \pm 2.0$ μM, $n_H = 0.92 \pm 0.03$). Saturation binding experiments indicated a non-competitive interaction such that SR 33589 (1 and 3 μM) and amiodarone (5 and 10 μM) reduced the B_{max} of [^{125}I]-(-)-CYP binding without any effect on the K_D. Kinetic studies showed that the rate of association of [^{125}I]-(-)-CYP was unchanged while the rate of dissociation was increased both in the presence of SR 33589 (10 μM) and amiodarone (30 μM).

3 Under the same conditions, the receptor stimulated adenylate cyclase activity was inhibited in a dose-dependent, but non-competitive manner, by SR 33589 (isoprenaline-, glucagon- and secretin-stimulated enzyme inhibited 50% at 6.8 ± 0.6 μM, 31 ± 10 μM and 12 ± 3 μM, respectively) while the basal, GTP- and Gpp(NH)p-stimulated enzyme was inhibited by 5-10% and the NaF and forskolin-stimulated enzyme by 50% at 500 μM. Amiodarone exhibited a similar pattern of inhibition.

4 After chronic oral treatment (50, 100, 150 mg kg^{-1} per day, 14 days), both SR 33589 and amiodarone produced a dose-dependent decrease in B_{max} without any effect on K_D as determined from [^{125}I]-(-)-CYP saturation experiments and a decrease of the isoprenaline- and glucagon-stimulated adenylate cyclase activity without any effect on basal enzyme activity or activity when stimulated by agents acting directly on regulatory catalytic units.

5 Unlike amiodarone, SR 33589 does not contain iodine substituents. Plasma levels of T_3, T_4 and rT_3 were unchanged after SR 33589 treatment except a decrease in T_4 level at the highest dose whilst the T_4 T_3 ratio and the level of rT_3 were dose-dependently increased by amiodarone treatment.

6 *In vitro*, SR 33589 and amiodarone were characterized as non-competitive β-adrenoceptor antagonists. Chronic treatment led to a down-regulation of the β-adrenoceptor; the down-regulation cannot be attributed to an indirect effect mediated by the thyroid hormones. To reconcile these opposing observations, we propose that SR 33589 and amiodarone interact with the β-adrenoceptor at a site close to the intracellular loops which are involved in the coupling with G_s and contain the phosphorylable sites.

Keywords: Amiodarone; SR 33589; antiarrhythmic drugs; cardiac β-adrenoceptor; adenylate cyclase; thyroid hormones; rat heart

Introduction

Amiodarone is known to be one of the most effective antiarrhythmic agents currently available for clinical use (Marcus *et al.*, 1981; Gill *et al.*, 1992). However, the exact pharmacological mechanisms responsible for amiodarone's antiarrhythmic actions are not settled. Amiodarone has a direct action on several ionic currents, including sodium, calcium and potassium fluxes (Gill *et al.*, 1992). These actions are interrelated in a complex manner, but are of prime importance for the activity of amiodarone. In addition, amiodarone does possess a partial antisympathetic activity (Charlier, 1970; Polster & Broekhuysen, 1976; Bacq *et al.*, 1976) which would certainly be an additional potential antiarrhythmic mechanism (Lubbe *et al.*, 1983). Striking similarities between the cardiac effects of amiodarone (e.g. action potential prolongation (Singh & Vaughan-Williams, 1970) and decrease in cardiac β-adrenoceptor density (Perret *et al.*, 1992) and those of hypothyroidism have led to the conclusion that the cardiac effects of amiodarone are due, at least in part, to an interaction between the drug and the thyroid hormones.

SR 33589 is a new antiarrhythmic agent, structurally related to amiodarone (Figure 1), with a similar pharmacological profile. SR 33589 is currently undergoing clinical trials. Like amiodarone, SR 33589 has been shown to prolong action potential duration in anaesthetized rats (Manning *et al.*, 1992) and to reduce significantly the incidence of life-threatening arrhythmias that arise upon reperfusion following a transient period of coronary artery occlusion in anaesthetized rats (Bruyninckx *et al.*, 1992) and during myocardial ischaemia in anaesthetized pigs (Finance *et al.*, 1994). Unlike amiodarone, SR 33589 contains no iodine substituent in its structure.

The purpose of the present study was to assess the ability of SR 33589, in comparison with amiodarone, to interact with the cardiac β-adrenoceptor. This was assessed by an *in vitro* rat cardiac membrane preparation following chronic treatment of the animals with SR 33589 and amiodarone. The rat was used as the effects of amiodarone chronic treatment on cardiac β-adrenoceptors and thyroid dysfunctions have been extensively studied in this animal species (Cohen-Armon *et al.*, 1984; Colvin *et al.*, 1989; Nokin *et al.*, 1983; 1986; Perret *et al.*, 1991; 1992).

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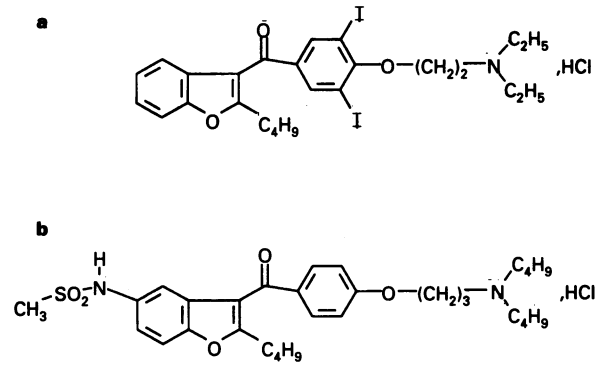

Figure 1 Chemical structures of amiodarone (a) and SR 33589 (b).

Methods

Animals and treatments

Male Wistar rats (200–250 g) (Sanofi-Pharma, Brussels, Belgium) were used. The animals received a standard diet with water freely available with a 12 h light/dark cycle. For the chronic treatment schedule (14 days), seven groups of ten rats were used. Animals received orally every 24 h either vehicle (5% arabic gum solution), SR 33589 (50 mg kg^{-1}, 100 mg kg^{-1} and 150 mg kg^{-1}) or amiodarone (50 mg kg^{-1}, 100 mg kg^{-1} and 150 mg kg^{-1}), and were weighed every two days. The rats were killed 24 h after the last administration by exsanguination under ether anaesthesia. Plasma was frozen at −20°C and the hearts rinsed in 0.9% w/v NaCl solution (saline), blotted, weighed, and were stored at −80°C until use.

Preparation of membranes

Membranes of ventricular tissues were prepared as described by Chatelain *et al.* (1980). Briefly, after thawing, the tissue samples were disrupted with a polytron tissue disruptor for 10 s at half speed and further homogenized with a glass-teflon homogenizer (5 strokes, 650 r.p.m.) in 20 volumes (w/v) of ice-cold Tris buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM dithioerythritol; pH 7.5). After filtration through two layers of cheese-cloth, the homogenates were centrifuged at 1,000 *g* for 10 min. The pellets were resuspended in the same initial volume of ice cold Tris-sucrose buffer (20 mM Tris-HCl, 250 mM sucrose, 10 mM $MgCl_2$, 2 mM dithioerythritol; pH 7.5). An equal volume of the Tris-sucrose buffer containing 2 M KCl was added dropwise. The suspensions were stirred continuously for 1 h at 2°C and then centrifuged at 48,000 *g* for 15 min. The resulting pellets were washed three times with the ice-cold Tris-sucrose buffer. The final pellets were resuspended in ice-cold Tris-sucrose buffer to a final concentration of 250 mg ml^{-1} original wet tissue weight, frozen in liquid nitrogen and store at −80°C. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin used as standard.

[^{125}I]-(−)-iodocyanopindolol binding assays

[^{125}I]-(−)-iodocyanopindolol ([^{125}I]-(−)-CYP) binding assays were performed essentially as described by Nokin *et al.* (1983). Membrane aliquots (0.16–0.24 mg ml^{-1}) were incubated for 60 min at 37°C in 50 mM Tris-HCl, 10 mM $MgCl_2$ at pH 7.4 with [^{125}I]-(−)-CYP (0.060 nM for competition and dissociation studies, 0.010–0.200 nM for saturation studies) in a total volume of 0.5 ml. In dissociation experiments, isotopic dilution was performed, by adding 2 μM (−)-alprenolol, after 1 h incubation. Incubations were carried out in Minisorb polypropylene tubes. Incubations were terminated by diluting the samples with 4 ml of incubation buffer at 37°C and rapid filtration over Whatman GF/C filters by use of a Brandel cell harvester. The filters were washed four times with the incubation buffer (37°C) and the bound radioactivity determined using a gamma counter (MAG 510, Berthold). Nonspecific binding was determined in the presence of 2 μM (−)-alprenolol.

Measurement of adenylate cyclase activity

Adenylate cyclase activity was assessed by the conversion of [α-^{32}P]-adenosine 5'-triphosphate ([^{32}P]-ATP) to [^{32}P]-adenosine 3':5' cyclic monophosphate ([^{32}P]-cyclic AMP) by modifications of the methods of Salomon *et al.* (1974). The incubation medium contained 30 mM Tris-HCl (pH 7.5), 0.5 mM ATP disodium salt, [^{32}P]-ATP (30 Ci $mmol^{-1}$) about 1 μCi per assay tube, 4 mM $MgCl_2$, 0.5 mM EGTA, 1 mM cyclic AMP, 1 mM theophylline, 10 mM creatine phosphate disodium salt, 0.1 mg ml^{-1} creatine kinase, 300–400 μg of membrane protein and the indicated substances. The reaction was started by addition of [^{32}P]-ATP to the incubation medium after equilibration for 2 min at 37°C. The assays were performed at 37°C in a final volume of 400 μl. At various intervals of time (2 min), the reaction was stopped by taking 60 μl of incubation medium in 0.5 ml of 0.1% cold sodium lauryl sulphate containing 0.5 mM ATP and 0.75 mM cyclic AMP supplemented with [^{3}H]-cyclic AMP (about 20,000 c.p.m., 23 Ci $mmol^{-1}$) for estimation of recovery. The [^{32}P]-ATP and [^{32}P]-cyclic AMP were separated by passage through Dowex AG-50WX4 (Biorad) and neutral alumina columns (aluminium oxide 90 active, Merck) (Salomon *et al.*, 1974) and assessed by scintillation spectroscopy (Beckman LS 3801). The reaction was linear with membrane protein content over the range used and for up to 10 min after the addition of [^{32}P]-ATP. The recovery of cyclic AMP was found to be between 65–70%.

Measurement of plasma thyroid hormone

Plasma thyroxine (T_4), triiodothyronine (T_3) and reverse triiodothyronine (rT_3) were measured with conventional RIA kits (T_4: Total T_4 double antibody, Diagnostic Products Corporation; T_3: Total T_3 double antibody, Diagnostic Products Corporation; rT_3: Biodata reverse T_3, Cat N° 10834, Serono Diagnostics).

Determination of SR 35589 and amiodarone contents in plasma and cardiac tissue

The SR 33589 and amiodarone content in plasma and cardiac tissue were assessed by a sensitive high performance liquid chromatographic assay, as described by Pourbaix *et al.* (1985) with minor modifications. The column used was a Lichrochart packed with Lichrospher 100 RP 18 (particle size 5 μm, 125 mm, 4.0 mm i.d.; Merck). A guard column Lichrosorb RP 18 (particle size 10 μm, 30 mm, 4.0 mm i.d.; Merck) was used to increase column life. The mobile phase consisted of acetonitrile, water and diethylamine (90/9.96/0.04) flowing at 1.5 ml min^{-1}. Ultraviolet detection was performed at 254 nm (amiodarone) and 280 nm (SR 33589). Calibration curves were established in the range 0.007–1.69 nmol for SR 33589 and 0.073–1.47 nmol for amiodarone. The limit of detection was 0.15 nmol g^{-1} cardiac tissue for each drug.

Analysis of data and statistics

Competition curves were generated by the use of at least ten concentrations in duplicate of competing drug. The IC_{50} (inhibitory concentration) and Hill coefficient (n_H) were estimated by the logit transformation (Hafner *et al.*, 1977). The K_i (inhibition constant), where appropriate, and the EC_{50} (effective concentration) were obtained from the equation of Cheng & Prusoff (1973). Equilibrium saturation parameters (K_D and B_{max}), of assay points carried out in duplicate, were determined by a method of computer-assisted nonlinear regression, based on the Clark equation (McIntosh, 1984) or by Scatchard

analysis (Scatchard, 1949). Dissociation kinetic constants (k_{-1}, k_{-2}) were determined by a method of computer-assisted non linear regression according to a two-site model (McIntosh, 1984). Regression lines were drawn by the method of Least Squares. The results are expressed as the mean ± s.e.mean. Standard statistical tests were used: Dixon's test (Dixon, 1951), analysis of variance: Bartlett test, Dunnett's multiple comparison test or Kruskal-Wallis test from Data Analysis. $P<0.05$ was considered to be statistically significant.

Materials

SR 33589 (2-n-butyl 3-[4-(3-di-n-butylamino-propoxy)-benzoyl]5-methylsulphonamido benzofuran hydrochloride) and amiodarone [2-butyl-3(3,5-diiodo-4-β-diethyl-aminoethoxybenzoyl)-benzofuran: Cordarone, Labaz] were synthesized in our Chemical Department.

Adenosine 5'-triphosphate (ATP) disodium salt, (±)-propranolol, (−)-alprenolol, (+)-alprenolol, secretin porcine (synthetic) were purchased from Sigma Chemical Co. (U.S.A.). Creatine phosphate disodium salt, creatine kinase, adenosine 3':5'-cyclic monophosphate (cyclic AMP) free acid, GTP, Gpp(NH)p and 1,4-dithioerythritol were purchased from Boehringer-Mannheim. EGTA and (±)-isopropylarterenol (isoprenaline) hydrochloride were purchased from Serva (Germany). Glucagon hydrochloride was obtained from S.A. Novo Nordisk Pharma N.V. (Belgium). Forskolin (Coleus forskohlii) was purchased from Calbiochem (U.S.A.). All other chemicals used were purchased from Merck (Germany) and were of the highest purity available. (−)-3-[^{125}I]-Iodocyanopindolol (≈74 Bq mmol^{-1}) and [8-^{3}H]-adenosine 3':5' cyclic phosphate ([^{3}H]-cyclic AMP) ammonium salt (851 GBq mmol^{-1}) were purchased from Amersham International plc (U.K.), [α-^{32}P] - adenosine 5' - triphosphate tetra(triethylammonium) salt (1.11 TBq mmol^{-1}) was purchased from New England Nuclear Du Pont (U.S.A.). Radioligands were stored at −20°C.

SR 33589, amiodarone, (±)-propranolol, (+)-alprenolol and (−)-alprenolol were dissolved in dimethylsulphoxide (DMSO) at a stock concentration of 5 mM and subsequent dilutions made in DMSO. The concentration of assay DMSO never exceeded 2% vol. in [^{125}I]-(−)-CYP binding assays and 1% vol. in adenylate cyclase assays. These final concentrations of DMSO were without effect on the assays. Forskolin was dissolved in methanol at a stock concentration of 1 mM and subsequent dilutions made in assay buffer. The concentration of ethanol in each assay never exceeded 0.8% vol. All other drugs were dissolved in assay buffer. (±)-Isoprenaline was prepared freshly in distilled water containing 1 mM ascorbic acid to inhibit photoxidation. Stock solutions were stored at −20°C.

Results

Effects of SR 33589 and amiodarone on [^{125}I]-(−)-CYP binding parameters

The binding of [^{125}I]-(−)-CYP to rat cardiac membranes at 37°C was saturable, reversible and of high affinity. Iterative non-linear analysis of the binding isotherms indicated a single class of high affinity sites with a K_D of 67 ± 1 pM, and a B_{max} of 21.1 ± 1.0 fmol mg^{-1} protein ($n=3$). In competition experiments (Figure 2), SR 33589 and amiodarone were able to displace [^{125}I]-(−)-CYP with a low affinity (IC_{50} 1.76 ± 0.36 μM, n_H 0.93 ± 0.06 for SR 33589; IC_{50} 8.65 ± 2.04 μM, n_H 0.92 ± 0.03 for amiodarone, n = 4). Reference β-adrenoceptor antagonists displayed high affinity for the [^{125}I]-(−)-CYP site (IC_{50} 11.6 ± 1.1 nM, n_H 0.86 ± 0.04 for (±)-propranolol; IC_{50} 5.3 ± 0.6 nM, n_H 0.85 ± 0.02 for (−)-alprenolol, $n=3$).

Saturation binding experiments carried out with [^{125}I]-(−)-CYP in the presence and absence of either SR 33589 or amiodarone (Figure 3) indicated that the two compounds caused a decrease in binding capacity (B_{max}) without any effect on binding affinity (K_D) (control, K_D 0.067 ± 0.001 nM, B_{max} 21.1 ± 1.0 fmol mg^{-1} protein; in the presence of 3 μM SR 33589, K_D 0.062 ± 0.003 nM, B_{max} 6.2 ± 0.8 fmol mg^{-1} protein; or 5 μM amiodarone, K_D 0.071 ± 0.007 nM, B_{max} 10.3 ± 0.1 fmol mg^{-1} protein, $n=3$). In both cases, these results were indicative of a non-competitive interaction at the [^{125}I]-(−)-CYP binding site.

In order to characterize the interaction of SR 33589 and amiodarone further, the effect of the compounds on [^{125}I]-(−)-CYP dissociation kinetics were examined. The concentrations of SR 33589 and amiodarone used in these experiments were 3 to 5 fold greater than their IC_{50} values for this site. Figure 4 illustrates the rate of dissociation of [^{125}I]-(−)-CYP from the β-adrenoceptor at equilibrium in the absence and presence of SR 33589 or amiodarone. As described previously (Hoyer *et al.*, 1982), the dissociation of [^{125}I]-(−)-CYP was biphasic with a slow component (k_{-1} 3.70 ± 0.34 × 10^{-3} min^{-1} representing ≈70% of the total number of binding sites, $n=4$) and a fast

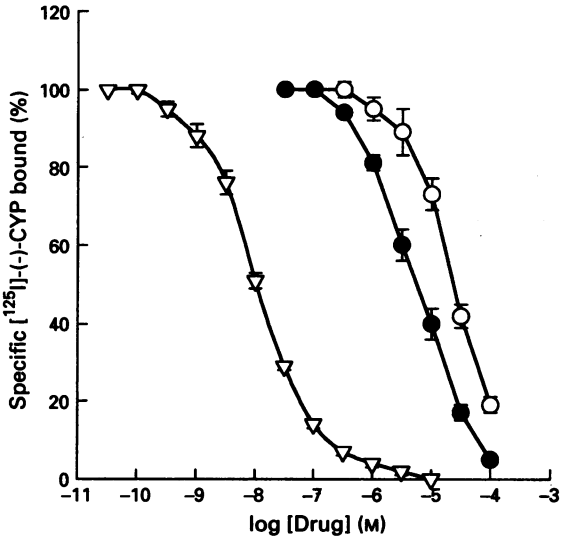

Figure 2 The effect of SR 33589 (●), amiodarone (○) and (±)-propranolol (▽) on [^{125}I]-(−)-iodocyanopindolol specific binding to rat cardiac membranes. Each point represents the mean ± s.e.mean of 3–4 separate experiments performed in duplicate. Corresponding affinity and pseudo Hill coefficient values are given in the text.

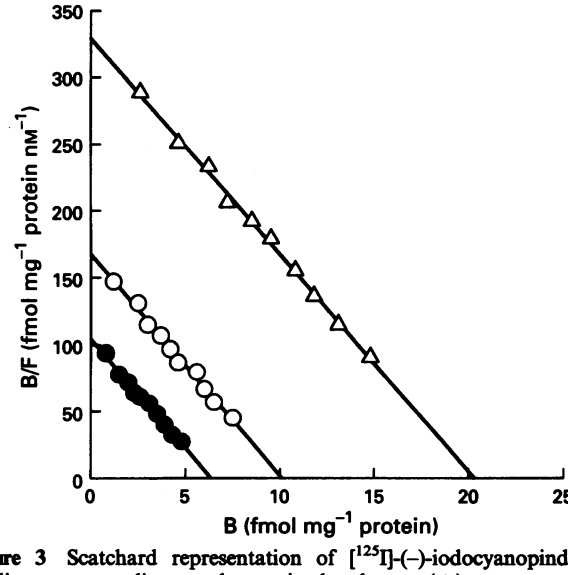

Figure 3 Scatchard representation of [^{125}I]-(−)-iodocyanopindolol binding to rat cardiac membranes in the absence (△) or presence of 3 μM SR 33589 or 5 μM amiodarone (○). B_{max} and K_D values are given in the text.

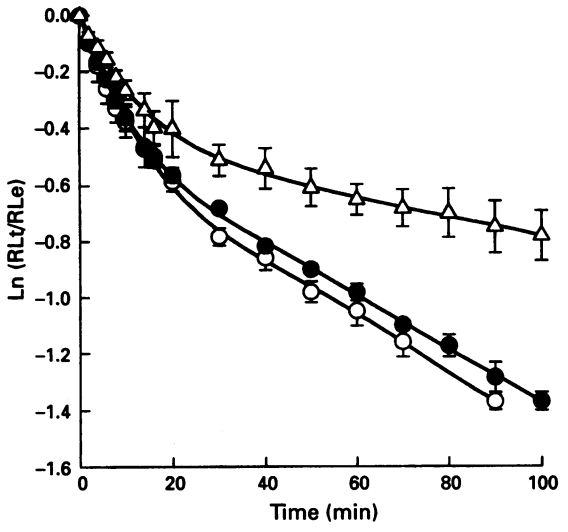

Figure 4 The effect of SR 33589 and amiodarone on the rate of [^{125}I]-(−)-iodocyanopindolol dissociation from rat cardiac membranes initiated by 2 μM (−)-alprenolol alone (control, △) or in the presence of 10 μM SR 33589 (●) or 30 μM amiodarone (○). LR_t = complex ligand receptor at a given time of dissociation process; LR_e = complex ligand receptor at equilibrium, before starting the dissociation. Each point represents the mean ± s.e.mean of 3–4 separate experiments performed in triplicate. The biphasic curves indicate two sites. The calculated k_{-1} and k_{-2}, related to the high and low affinity sites, are given in the text.

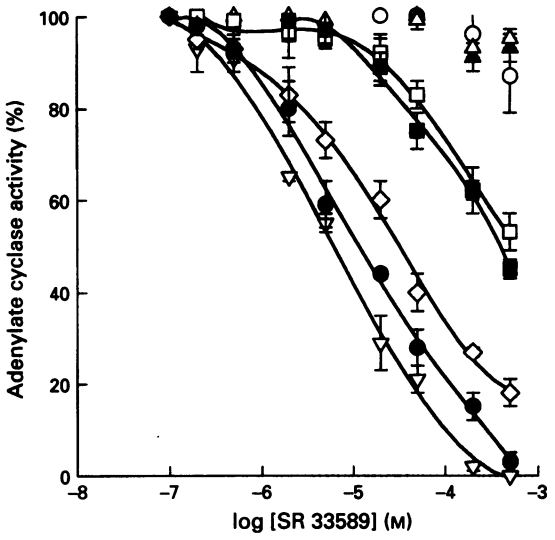

Figure 5 The effect of SR 33589 on basal (○) or 10 μM GTP (▲)-, 100 μM Gpp(NH)p (△)-, 1 mM isoprenaline (▽)-, 10 μM glucagon (◇)-, 3 μM secretin (●)-, 10 mM NaF (□)-, and 100 μM forskolin (■)-stimulated adenylate cyclase activity in rat cardiac membranes. Isoprenaline, glucagon and secretin were added in the presence of 10 μM GTP. GTP-, Gpp(NH)p-, sodium fluoride (NaF)- and forskolin-stimulated activities were considered as the increase in activity above basal levels. Isoprenaline-, glucagon- and secretin-stimulated activities were considered as the increase in activity above that seen with GTP alone. Each point represents the mean ± s.e.mean of 3 separate experiments. The calculated IC_{50} values for the effect of SR 33589 on isoprenaline-, glucagon-, and secretin-stimulated adenylate cyclase activity are given in the text.

component (k_{-2} $90.1 \pm 8.8 \times 10^{-3}$ min^{-1} representing ≈30% of the total number of binding sites, $n = 4$). SR 33589 and amiodarone were without any effect on the fast component (k_{-2} $114.9 \pm 19.1 \times 10^{-3}$ min^{-1}, $n = 3$ and k_{-2} $81.3 \pm 2.4 \times 10^{-3}$ min^{-1}, $n = 3$ for SR 33589 and amiodarone respectively, the proportion of binding sites remaining constant around 30%) but increased the slow component (k_{-1} $9.06 \pm 0.13 \times 10^{-3}$ min^{-1} and k_{-1} $10.05 \pm 1.31 \times 10^{-3}$ min^{-1} for SR 33589 and amiodarone respectively).

Effects of SR 33589 and amiodarone on adenylate cyclase activity

Using the same membrane preparation, the activity of the basal and stimulated adenylate cyclase was measured. The following activities, expressed as pmol cyclic AMP min^{-1} mg^{-1} protein, were obtained at the indicated concentrations of each stimulant: basal 15.6 ± 0.4, 10 μM GTP 23.4 ± 2.1, 100 μM Gpp(NH)p 105 ± 6, 10 mM NaF 114 ± 9, 100 μM forskolin 337 ± 25, 1 mM isoprenaline 57.5 ± 3.5, 10 μM glucagon 42.2 ± 2.7, 3 μM secretin 34.3 ± 0.5.

SR 33589 (1–500 μM) and amiodarone (1–500 μM) alone or in the presence of 10 μM GTP did not increase the corresponding adenylate cyclase activity. The activity of adenylate cyclase was inhibited in a dose-dependent manner by SR 33589 (Figure 5) and amiodarone (data not shown). With respect to the various stimuli, a similar pattern of inhibition was observed for the two compounds (Table 1): isoprenaline ≈ secretin ≈ glucagon > NaF ≈ forskolin > GTP, Gpp(NH)p ≈ 0. Stimulation of the cardiac adenylate cyclase by isoprenaline in the presence of various fixed amounts of SR 33589 (Figure 6) produced a dose-related decrease of the maximal activity of the enzyme (maximum response reduced by 87 ± 8% of control by 50 μM SR 33589) with no change in EC_{50} values. Similar results were obtained previously with amiodarone (Gagnol *et al.*, 1985). In both cases, these results were indicative of a selective, but non-competitive, interaction at the β-adrenoceptor site.

Table 1 Effects of SR 33589 and amiodarone on basal and stimulated adenylate cyclase activity

Adenylate cyclase activity		*SR 33589* (% inhibition) at 500 μM		*Amiodarone* (% inhibition) at 500 μM
Basal		13 ± 8		0 ± 8
+ GTP		7 ± 3		8 ± 2
+ Gpp(NH)p		5 ± 2		11 ± 3
+ NaF		47 ± 4		26 ± 6
+ Forskolin		58 ± 5		5 ± 2
	IC_{50} (μM)	n_H	IC_{50} (μM)	n_H
+ Isoprenaline	6.8 ± 0.6	0.61 ± 0.01	18 ± 7	0.56 ± 0.2
+ Glucagon	31 ± 10	0.60 ± 0.06	65 ± 10	0.65 ± 0.04
+ Secretin	12 ± 3	0.67 ± 0.04	9 ± 3	0.63 ± 0.04

Each value represents the mean ± s.e.mean of 3–6 experiments.

Effects of chronic treatment with SR 33589 and amiodarone on [^{125}I]-(−)-CYP binding and adenylate cyclase activity

Analysis of saturation curves of [^{125}I]-(−)-CYP binding to cardiac membranes from vehicle and SR 33589 or amiodarone treated rats (50, 100 and 150 mg kg^{-1} day^{-1}, 14 days) indicated a single class of high affinity sites. Treatment produced a dose-related decrease in B_{max} without any effect on K_D (Table 2). The maximum decrease was 50% and 45% for SR 33589 and amiodarone respectively. Measurement of basal and stimulated-adenylate cyclase activity (Table 3), using the same membranes, indicated a dose-related decrease in the activity of isoprenaline-stimulated enzyme. The maximum decrease, 31% and 37% for SR 33589 and amiodarone respectively, was selective since the basal and GTP-, Gpp(NH)p-, NaF-, forskolin-stimulated adenylate cyclase activity was not modified.

Plasma thyroid hormone levels and cardiac drug concentration

Amiodarone treatment produced a dose-related increase in plasma rT_3 levels and T_4/T_3 ratios. These results were those generally expected in rat after amiodarone administration (Wiegand *et al.*, 1986; Ceppi & Zaninovich, 1989; Perret *et al.*, 1991). In contrast, SR 33589 treatment either had no effect on plasma thyroid hormones (50 and 100 mg kg^{-1} day^{-1}) or induced a decrease in the T_4/T_3 ratio (150 mg kg^{-1} day^{-1}) resulting from a decrease in T_4 and probably due to treatment intolerance (Table 4). The body weight of this latter group decreased during the treatment (−20% at the end of the treatment), while the other regimens for both SR 33589 and amiodarone did not modify the body weight as compared to the control group.

Cardiac SR 33589 concentrations, expressed as nmol mg^{-1} protein, were 0.006±0.002 (n=8), 0.013±0.003 (n=9) and 0.034±0.014 (n=10) for the daily dose of 50, 100 and 150 mg kg^{-1}. Cardiac amiodarone concentrations, expressed as nmol mg^{-1} protein, were 0.068±0.019 (n=10), 0.326±0.076 (n=10) and 0.461±0.096 (n=10) for the daily dose of 50, 100 and 150 mg kg^{-1}. From the known protein quantity per g of rat heart, and assuming a cardiac tissue density of 1, the compound concentrations expressed as μM were 0.12, 0.26 and 0.68 for SR 33589 and 1.4, 6.5 and 9.2 for amiodarone for the daily dose of 50, 100 and 150 mg kg^{-1}. For both compounds, the cardiac drug concentrations increased with the dose. The SR 33589 cardiac concentrations were roughly 10 times lower than the amiodarone cardiac concentrations. The amiodarone concentrations were very similar to those measured previously with similar treatment schedules (Nokin *et al.*, 1983; Plomp *et al.*, 1985).

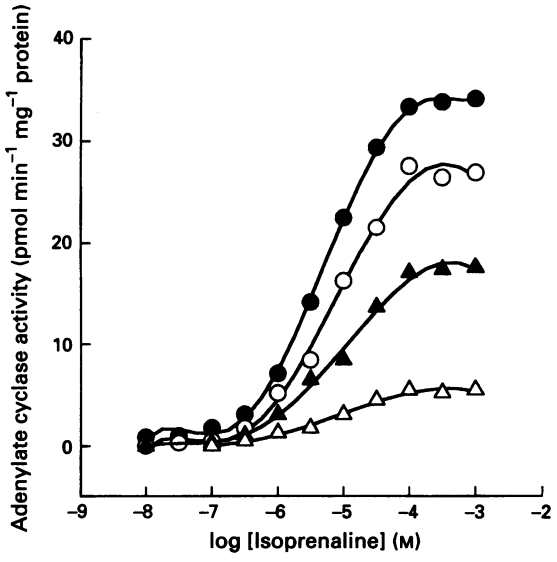

Figure 6 The effect of SR 33589 on maximal activation of adenylate cyclase by isoprenaline measured in the absence or in the presence of 2 μM, 10 μM or 50 μM SR 33589. Effective concentrations (EC_{50}) were estimated as follows: control (●) 4.2 μM; 2 μM SR 33589 (○) 5.3 μM; 10 μM SR 33589 (▲) 7.7 μM; 50 μM SR 33589 (△) 5.9 μM.

Table 2 Effects of chronic treatment with SR 33589 and amiodarone on the specific binding of [^{125}I]-(−)-CYP to rat cardiac membranes

		Binding parameters		
Treatment	*Dose* (mg kg^{-1})	K_D (pM)	B_{max} (fmol mg^{-1} protein)	n
Vehicle	–	87±4	21.6±0.7	10
SR 33589	50	91±2	17.2±1.0***	10
	100	90±3	15.3±1.8**	9
	150	89±3	10.8±1.0***	10
Amiodar-	50	88±3	17.7±1.3*	10
one	100	93±3	12.7±0.9***	10
	150	86±2	11.9±0.7***	10

Each drug was administered by oral route every 24 h for 14 days. The rats were killed 24 h after the last administration. Each value represents the mean±s.e.mean of n experiments per group with an animal per experiment.
*P<0.05; **P<0.01; ***P<0.001 when compared with the vehicle-treated group.

Table 3 Effects of chronic treatment with SR 33589 and amiodarone on basal and stimulated adenylate cyclase activity in rat cardiac membranes

		Adenylate cyclase activity (pmol cyclic AMP min^{-1} mg^{-1} protein)				
Treatment	*Dose* (mg kg^{-1})	*+GTP* (10 μM)	*+Gpp(NH)p* (100 μM)	*+Forskolin* (100 μM)	*+Isoprenaline* (1 mM)	n
Vehicle	–	25.8±1.7	98±4	442±14	60.3±1.6	10
SR 33589	50	23.0±1.4	95±5	426±31	51.0±1.9**	10
	100	24.3±0.6	99±4	490±20	51.0±2.4**	9
	150	23.5±1.1	93±6	440±9	41.4±3.7***	10
Amiodarone	50	24.9±1.3	98±3	458±21	55.0±6.1	10
	100	23.3±2.0	86±6	402±32	43.8±4.5**	10
	150	22.7±1.0	84±2	406±28	37.8±3.2***	10

Each drug was administered by oral route every 24 h for 14 days. The rats were killed 24 h after the last administration. Each value represents the mean±s.e.mean of n experiments per group with an animal per experiment.
**P<0.01; ***P<0.001 when compared with the vehicle-treated group.

Table 4 Effects of chronic treatment with SR 33589 and amiodarone on plasma thyroid hormone levels in rat

Treatment	Dose (mg kg^{-1})	T_4 (ng ml^{-1})	T_3 (ng ml^{-1})	rT_3 (pg ml^{-1})	T_4/T_3 ratio	n
Vehicle	–	54±2	0.93±0.03	60±4	58±1	10
SR 33589	50	56±4	0.98±0.05	61±7	57±2	10
	100	49±3	0.76±0.04	63±6	62±1	9
	150	35±3***	0.76±0.06	48±4	46±2***	10
Amiodarone	50	62±3	0.94±0.04	133±7***	66±4	10
	100	61±3	0.81±0.05	210±7***	69±3**	10
	150	50±3	0.73±0.03**	222±15***	75±1***	10

Each drug was administered by oral route every 24 h for 14 days. The rats were killed 24 h after the last administration. Each value represents the mean±s.e.mean of n experiments per group with an animal per experiment.
**$P<0.01$; ***$P<0.001$ when compared with the vehicle-treated group.

Discussion

The results of this study are consistent with previous data that demonstrated *in vitro* the non-competitive interaction between amiodarone and the β-adrenoceptor (Nokin *et al.*, 1983; Gagnol *et al.*, 1985) and, after chronic treatment, the existence of a reduced density of the cardiac β-adrenoceptors (Nokin *et al.*, 1983; Venkatesh *et al.*, 1986; Yin *et al.*, 1992). SR 33589, a compound structurally related to amiodarone, has been shown to possess similar properties.

In equilibrium binding studies, displacement of [^{125}I]-(–)-CYP by amiodarone and SR 33589 has the characteristics of an apparent competitive inhibition (complete displacement of the specific binding and $n_H \approx 1$). However, saturation experiments performed in the absence and the presence of either amiodarone or SR 33589 demonstrated a decrease in B_{max} without any effect on K_D which is characteristic of a non-competitive inhibition. These effects were described previously for amiodarone (Nokin *et al.*, 1983). SR 33589, which has similar interactions, is however more active than amiodarone. To characterize fully the interaction between amiodarone or SR 33589 and the β-adrenoceptor using radioligand binding techniques, kinetics studies were performed. This is the first time the results of such studies have been described. Both compounds increased the rate of dissociation of [^{125}I]-(–)-CYP which suggests the existence of negative co-operativity between the binding sites, since occupation of the sites with unlabelled ligand would reduce the overall affinity of the binding site population, thus resulting in an increased dissociation rate. In the studies of adenylate cyclase activity, inhibition of isoprenaline-stimulated enzyme activity by amiodarone and SR 33589 had the characteristics of an apparent non-competitive inhibition. The IC_{50} values for either SR 33589 or amiodarone obtained from binding and adenylate cyclase experiments are in close agreement. Thus with respect to the β-adrenoceptor, it is concluded that the two compounds display a similar non-competitive-interaction. This interaction is selective with respect to the catalytic unit and the G-protein, the activity of those are not or only slightly affected as compared to the isoprenaline-stimulated enzyme. On the other hand, the interaction is non-selective with respect to two other receptors (glucagon and secretin/VIP) since the stimulation of adenylate cyclase by the appropriate hormone (glucagon or secretin) is inhibited to a similar extent and with a similar potency by amiodarone and SR 33589. In addition to the β-adrenoceptor, the interaction between amiodarone and various enzymes, receptors or channels associated with the cardiac sarcolemma and other cytoplasmic membranes (for example brain and erythrocyte membranes) have been studied in great detail (Cohen-Armon *et al.*, 1984, Nokin *et al.*, 1986; Sheldon *et al.*, 1989; Chatelain *et al.*, 1989; Colvin *et al.*, 1989; Wagner *et al.*, 1990). Whatever the protein studied, the overall conclusion is that the binding and/or enzymatic data do not comply with a simple model of competitive inhibition, but rather with allosteric inhibition. Due to the highly lipophilic nature of amiodarone, a 'membrane pathway' which implies firstly partitioning into and diffusion through the plasma membrane, follow by binding at a specific site in the target protein, has been proposed recently (Herbette *et al.*, 1988; Mason *et al.*, 1991). The data obtained in this present study with SR 33589 suggest that this agent also has a similar pattern of interaction with the membrane proteins.

Pharmacological findings of both *in vivo* and *in vitro* studies led to the classification of amiodarone as a non-competitive β-blocker (Bauthier *et al.*, 1976, Polster & Broekhuysen, 1976). Binding and enzymatic studies confirmed this initial characterization. However, continuous blockade of the β-adrenoceptor with amiodarone produces a down-regulation of the cardiac β-adrenoceptor without any effect on the catalytic unit and the G-protein of adenylate cyclase. In addition to the present study, this has been observed in rat (Nokin *et al.*, 1983; Yin *et al.*, 1992), rabbit (Venkatesh *et al.*, 1986), pig (Gøtzsche, 1993) and man (Bjørnerheim *et al.*, 1991) but is still unexplained. This is directly opposite to what is expected since the continuous exposure to antagonists leads to an up-regulation of the cardiac β-adrenoceptor (Maisel *et al.*, 1987). Several explanations have been put forward to explain this atypical behaviour. Firstly the agent may have some intrinsic agonist activity. *In vitro* and *in vivo* studies failed to show any agonistic activity which could explain the β-adrenoceptor down-regulation. Secondly the compound is highly hydrophilic and is retained in the membrane; the decrease in β-adrenoceptor could be due to the presence of amiodarone. This could be the case for the high dosages (100 and 150 mg kg^{-1}) of amiodarone since this is the level of amiodarone in cardiac tissue at which *in vitro* studies showed non-competitive interactions with the β-adrenoceptor. However the cardiac amiodarone level at 50 mg kg^{-1} day^{-1} is not high enough to explain using the same mechanism a decrease of 18% of the β-adrenoceptor density. This conclusion is reinforced by the findings with SR 33589. For this agent, the tissue levels are well below the concentrations needed to decrease the total number of binding sites and the isoprenaline-stimulated enzyme. Thirdly the down-regulation of the cardiac β-adrenoceptor is often ascribed to an indirect effect due to the actions of amiodarone on thyroid hormone levels (Perret *et al.*, 1992; Yin *et al.*, 1992) leading to hypothyroidism and thus inducing a decrease in β-adrenoceptor density (Maisel *et al.*, 1987). However SR 33589, which does not contain an iodine substituent in its structure and does not produce a hypothyroid-like state, also induces a down-regulation in the number of the cardiac β-adrenoceptors. This indicates that the effects of amiodarone on thyroid hormones is not the main mechanism to promote β-adrenoceptor down-regulation. Finally, the fact that the stimulation of adenylate cyclase via three independent receptors, i.e. the β-adrenoceptor, glucagon and secretin receptor, is selectively inhibited suggests an additional mechanism of interaction. The β-adrenoceptor, glucagon and secretin receptors are members of the large superfamily of G protein-coupled receptors

(Dohlman *et al.*, 1991; Burbach & Meijer, 1992) which exhibit high degrees of amino acid conservation especially in regions implicated in ligand binding and interactions with G proteins. The results contained in this study indicate that amiodarone and SR 33589 interact with the β-adrenoceptor in a site which is not the binding site of the β-adrenoceptor antagonist. It is proposed that amiodarone and SR 33589 interact with the region of the β-adrenoceptor involved in the coupling with G protein. This proposed region includes the seventh transmembrane domain and proximal part of the cytoplasmic carboxyl terminus of virtualy all G protein-coupled receptors (Dohlman *et al.*, 1991; Burbach & Meijer, 1992). This region includes also a highly conserved tyrosine (Barak *et al.*, 1994) and several serine residues (Hausdorff *et al.*, 1991) involved in β-adrenoceptor regulation. An interaction of amiodarone and SR 33589 at a site which could be at the interface between the β-adrenoceptor and the G-protein would explain the apparently contradictory effects of two compounds: non-competitive antagonist *in vitro* and down-regulation after chronic treatment. Furthermore, the binding of the two antiarrhythmic agents at such a site could explain the inhibition of the glucagon- and secretin-stimulated cardiac adenylate cyclase since the regions implicated in the coupling with the G-protein are highly conserved.

The structure and location of amiodarone within the lipid bilayer has been studied in detail (Trumbore *et al.*, 1988; Chatelain & Brasseur, 1989; Jendrasiak *et al.*, 1990; Chatelain, 1991). It has been concluded that the drug penetrates into the acyl chains of the lipids with the amino-side chain positively charged at neutral pH values orientated towards the polar head of the lipids and the external aqueous phase. Preliminary data obtained with SR 33589 suggests a similar orientation (Chatelain, unpublished data). Such a location and an orientation in the bilayer would contribute to the interaction with the seventh hydrophobic transmembrane domain of the β-adrenoceptor and the cytoplasmic terminus at the lipid-water interface.

In conclusion, in this study we have characterized the interactions of amiodarone and SR 33589 with the cardiac β-adrenoceptor. The two compounds have a similar pattern of interaction: non-competitive antagonism *in vitro* and a down-regulation in receptor number after chronic treatment. The major differences being that SR 33589 does not induce a hypothyroid-like state unlike amiodarone, and that the cardiac levels of SR 33589 are 10 times lower than those of amiodarone, whilst the β-adrenoceptor density is decreased by the same amount. From the comparison of the effects of the two compounds, a mechanism of action of amiodarone on the β-adrenoceptor has been proposed. It is suggested that amiodarone binds on the β-adrenoceptor in the region involved in the coupling with the G-protein.

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