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CARDIAC β-ADRENOCEPTOR MODULATION BY AMIODARONE

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Abstract—β-Antiadrenergic properties are part of the pharmacological characteristics of amiodarone. In the present study, the action of amiodarone on rat-heart β-adrenoceptors was investigated. [^{125}I]Cyanopindolol (CYP) was used to label β-adrenoceptors in crude rat-heart microsomes. In competition binding experiments, amiodarone up to 10^{-6} M did not displace [^{125}I]CYP from cardiac β-adrenergic receptors. The effects of amiodarone on the number and affinity for [^{125}I]CYP of β-adrenoceptors were evaluated in saturation experiments. *In vitro* exposure of cardiac microsomes to 10^{-5} M amiodarone did not modify these parameters. At higher concentrations the β-receptor number decreased while the affinity for [^{125}I]CYP was not affected. *In vivo* experiments showed a significant decrease in β-adrenoceptor density after a single oral dose of 50 mg/kg amiodarone. In chronically treated animals, the same decrease in β-receptor number was observed 24 hr after the last administration of the drug. 5′-Nucleotidase activity, another specific marker of the plasma membrane, was unaffected by the treatment. These results suggest that part of the β-adrenergic antagonism of amiodarone is due to a decrease in the β-adrenoceptor density at the surface of the myocardial cell.

Amiodarone [2-butyl-3-(3,5-diiodo-4-β-diethylaminoethoxybenzoyl)-benzofuran: cordarone®, Labaz] is a potent antianginal and antiarrhythmic drug (see [1] for a review). The main pharmacological properties of amiodarone which account for its beneficial effect in the treatment of angina pectoris include heart-rate decrease, lowering of myocardial oxygen consumption and α- as well as β-adrenergic antagonism [2]. Amiodarone has been shown to antagonize the effects of catecholamines and sympathetic stimulation through a non-competitive mechanism of inhibition [3, 4]. The present experiments were carried out in order to further document the *in vitro* and *in vivo* actions of amiodarone at β-adrenergic receptor sites in cardiac membranes. Binding studies were performed using the new, highly specific β-adrenergic antagonist (±)-[^{125}I]iodocyanopindolol ([^{125}I]CYP) [5, 6] as a radioligand.

MATERIALS AND METHODS

Animals and treatment. Male Sprague–Dawley rats (300–350 g) were used in all studies. For acute and chronic treatment amiodarone was administered by gastric intubation as a 2.5% (w/v) aqueous solution of the hydrochloride at a dose of 50 mg/kg. Groups of matched control rats were treated similarly, receiving only water. In chronically treated animals, amiodarone was administered daily for 4 weeks, and hearts were excised and processed for cardiac membrane isolation 24 hr after the last drug administration. In acute experiments, hearts were excised 4 hr after drug administration when the maximal myocardial drug level was reached. Myocardial amiodarone concentrations were measured by a sensitive high-pressure liquid chromatographic assay [7].

Membrane preparation. The method of Minneman *et al.* [8] was used. Briefly, rats were killed by decapitation and hearts were rapidly excised, cleaned of non-myocardial tissues and homogenized in 20 volumes of ice-cold 154 mM NaCl containing 5 mM Tris–HCl, pH 7.4, using an Ultra Turrax (Jahnke & Kunkel, Stauffen, F.R.G.) for 15 sec at half speed. The homogenate was filtered through two layers of medical gauze and centrifuged at 10,000 *g* for 10 min at 4°. The resulting pellets were washed once with 20 volumes of ice-cold incubation buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4) by resuspension and centrifugation, and finally resuspended in 20 ml of incubation buffer per g wet weight. Membrane suspensions were either immediately used to assay β-adrenergic receptors and 5′-nucleotidase activity or frozen in liquid nitrogen and stored at −80°. Protein concentration was determined by the method of Lowry *et al.* [9] using bovine serum albumin as standard.

β-Adrenergic binding assay. (±)-[^{125}I]iodocyanopindolol ([^{125}I]CYP) with a specific radioactivity higher than 2 Ci/μmole was obtained from the Radiochemical Centre (Amersham, U.K.). Membranes (150–200 μg protein) were incubated in the presence of [^{125}I]CYP in 0.5 ml of 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.4. Incubation was performed in disposable polypropylene tubes at 37° for 60 min, except in the experiment described in Fig. 2. At the end of the incubation period, 25 μl was taken for total count determination, and binding was stopped by the addition of 5 ml of incubation buffer maintained at 37° and immediate filtration

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through glass-fibre filters GF/C (Whatman, Maidstone, U.K.). Radioactivity on each filter, which was washed 4 times with 5 ml of buffer at 37°, was determined using a gamma counter (MAG 510, Berthold). Non-specific binding of [^{125}I]CYP was defined as the radioactivity bound to membranes in the presence of cold (−)-alprenolol (2 μM). Specific binding was defined as the total radioactivity minus the non-specific binding. Total tracer binding never exceeded 10% of the radioactivity offered.

The density of [^{125}I]CYP binding sites was determined by incubating the membranes (150–200 μg protein) in the presence of ten different concentrations of [^{125}I]CYP (from 25 to 500 pM). The equilibrium dissociation constant (K_D) and the density of receptor sites (B_{max}) were determined by Scatchard plots [10]. In competition binding experiments, membranes (150–200 μg protein) and [^{125}I]CYP (150,000–200,000 cpm; 90–120 pM) were incubated with 12–14 different concentrations of the competing agent, and specific binding was determined as described above.

5′-Nucleotidase assay. Nickel-sensitive 5′-nucleotidase activity was measured according to Campbell [11]. Briefly 150–200 μg membrane protein was incubated at 37° over a 20 min period in 1 ml of barbital buffer, pH 7.5, 1 mM $MnSo_4$, 1 mM AMP in the presence and absence of 10 mM $NiCl_2$. Reaction was terminated by adding 2 ml ice-cold 10% (w/v) trichloracetic acid, and inorganic phosphate release was measured by the method of Fiske and Subbarow [12]. 5′-Nucleotidase activity was taken as that activity inhibitable by 10 mM $NiCl_2$.

Statistical analysis. All measured parameters are reported as mean ± S.E.M. Statistical significance was calculated using a two-tailed Student's *t*-test, taking P = 0.05 as the limit of significance.

RESULTS

Binding characteristics of [^{125}I]CYP *to rat-heart membranes*

A typical saturation curve is shown in Fig. 1. [^{125}I]CYP binding was saturable and the Scatchard plot (inset) appeared linear ($r^2 = 0.98$), consistent with radiologand binding to an apparently homogeneous population of receptors. Non-specific binding was low, never exceeding 15% of the total bound fraction when measured at a concentration of free ligand near the K_D. Based on Scatchard analysis of saturation curves, the K_D for [^{125}I]CYP binding to rat-heart β-adrenergic receptor was 99 ± 6 pM and B_{max} was 25.1 ± 0.8 fmole/mg membrane protein ($n = 25$). The time-dependent association of [^{125}I]CYP to rat-heart membranes was studied in order to determine the optimal conditions for the binding assay (Fig. 2). The equilibrium for binding of [^{125}I]CYP was reached after about 60 min of incubation at 37° with a $t_{1/2}$ of about 10 min and remained stable for at least a further 90 min. When a high concentration of (−)-alprenolol (5 μM) was added after 30 min of incubation, dissociation of [^{125}I]CYP from the binding sites was observed. The dissociation reaction presented a biphasic profile as already shown with this radioligand in other membrane preparations of various origins [5, 6, 13]. As seen in Fig. 3, [^{125}I]CYP binding to rat-heart membrane showed stereospecificity, in that (−)-alprenolol was two orders of magnitude more potent than its (+)-stereoisomer in displacing the radioligand.

Effect of amiodarone added in vitro *on* [^{125}I]CYP *binding to rat-heart membranes*

Competition between amiodarone and [^{125}I]CYP for binding to β-adrenoceptors was tested by incu-

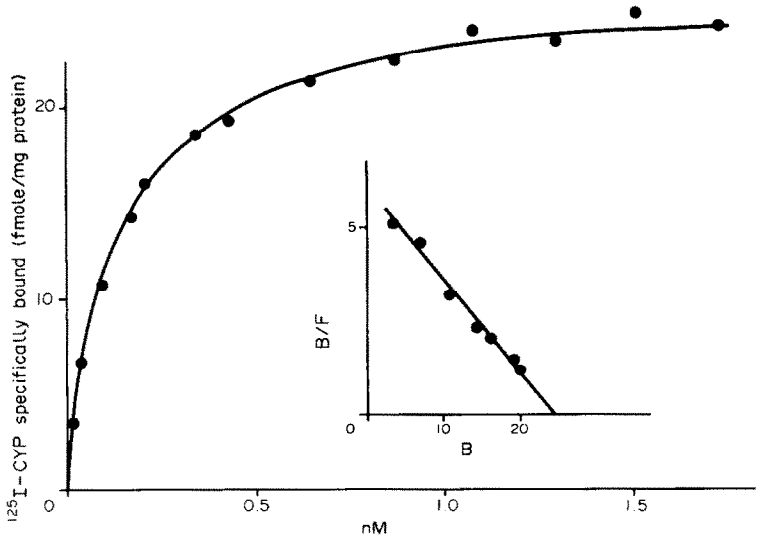

Fig. 1. Specific binding of [^{125}I]CYP to rat-heart membranes as a function of increasing [^{125}I]CYP concentrations. Binding was carried out as described in Materials and Methods at various concentrations of [^{125}I]CYP, ranging from 25 to 2000 pM. Specific binding was defined as the [^{125}I]CYP binding displaceable by 2 μM (−)-alprenolol. Data points represent means of duplicate determinations from a representative experiment. The K_D for [^{125}I]CYP was 106 pM and the maximal binding capacity was 24.7 fmole/mg protein. Scatchard analysis of the binding data revealed a straight line ($r^2 = 0.982$).

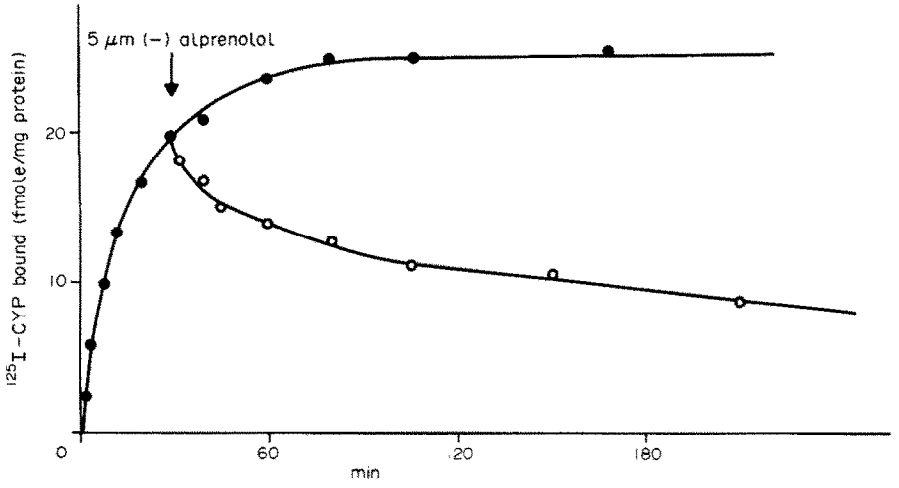

Fig. 2. Forward and reverse kinetics of [^{125}I]CYP binding to rat-heart membranes. Membranes (1.63 mg/ml) and [^{125}I]CYP (270 pM) were incubated in the presence or absence of 2 μM (−)-alprenolol at 37° (●——●). At the indicated time, the reaction was stopped by dilution with incubation buffer at 37° and specific binding was estimated as described in Materials and Methods. After 30 min of incubation (−)-alprenolol was added to the original incubation medium to a final concentration of 5 μM and specific binding was determined at subsequent time intervals after the addition of (−)-alprenolol (○——○). Data points represent means of duplicate determinations from one of three representative experiments.

bating cardiac membranes with varying concentrations of amiodarone and a fixed concentration of the radioligand, and subsequently determining the amount of [^{125}I]CYP bound. Figure 3 shows that up to 10^{-6} M amiodarone did not interfere with [^{125}I]CYP binding. At higher concentrations, amiodarone decreased radioligand binding in a dose-dependent manner. To determine whether this inhibition was competitive or non-competitive, we measured saturation binding isotherms in the presence and absence of amiodarone. Scatchard analysis of the saturation curves measured at different amiodarone concentrations showed that up to 10^{-4} M the apparent K_D for [^{125}I]CYP was not modified, while the maximal binding capacity (B_{max}) decreased progressively with increasing concentrations of the drug (Table 1). This result indicates that inhibition of [^{125}I]CYP binding to β-adrenergic receptors by amiodarone [which occurred at high concentrations (Fig. 3)] is non-competitive. 5′-Nucleotidase activity, another marker of the sarcolemma, was unaffected by amiodarone in the same range of concentrations.

Effect of in vivo *amiodarone administration on β-adrenoceptor density in rat-heart membranes*

In order to test if modifications in specific [^{125}I]CYP binding induced *in vitro* by amiodarone were also observed after *in vivo* administration, rats were

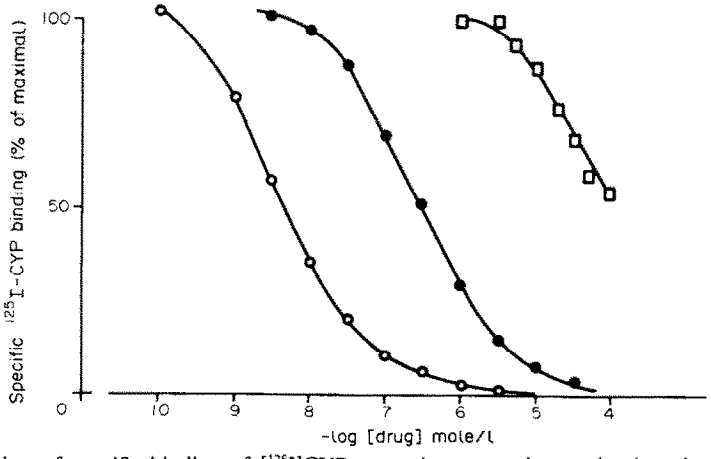

Fig. 3. Inhibition of specific binding of [^{125}I]CYP to rat-heart membranes by (−)-alprenolol, (+)-alprenolol and amiodarone. Membranes were incubated in the presence of [^{125}I]CYP (90–120 pM) and increasing concentrations of (−)-alprenolol (○——○), (+)-alprenolol (●——●) and amiodarone (□——□). After 60 min of incubation, specific binding was estimated as described in Materials and Methods. The results are expressed as the percentage of [^{125}I]CYP specifically bound and were the means of three experiments performed in triplicate.

Table 1. *In vitro* effects of amiodarone on binding parameters of [^{125}I]CYP to rat-heart membranes

Amiodarone concentrations (μM)	B_{max} (fmoles/mg protein)	K_D (pM)	5'-Nucleotidase activity (μmoles/hr × mg protein)
0	24.4 ± 1.7	97 ± 11	1.91
10	22.8 ± 2.4	95 ± 14	1.94
50	16.9 ± 2.0*	100 ± 9	1.91
100	12.1 ± 1.4*	95 ± 14	1.90

Rat-heart membranes were incubated in the presence or absence of amiodarone for 60 min. [^{125}I]CYP was added at various concentrations ranging from 20 to 200 pM, and specific binding was measured as described in Materials and Methods. 5'-Nucleotidase activity was measured at the end of the 60 min incubation period with amiodarone. B_{max} and K_D are expressed as the mean value ± S.E.M. of three experiments. 5'-Nucleotidase activity was only measured in two of these experiments.

* $P < 0.005$.

treated with a single oral dose of 50 mg/kg amiodarone and cardiac membranes were isolated 4 hr later. At that time, myocardial drug level reached 3.1 ± 0.5 μg/g fresh weight. On each heart, saturation binding isotherms of [^{125}I]CYP were measured (Table 2). [^{125}I]CYP affinity for β-adrenergic receptors was not modified by amiodarone treatment but a 16% decrease in maximal binding capacity of [^{125}I]CYP was repeatedly observed to a statistically significant degree. 5'-Nucleotidase activity, another marker of the sarcolemma, was unaffected by this treatment. Similar results were observed after chronic treatment at a daily oral dose of 50 mg/kg amiodarone for 4 weeks (Table 2). In these experiments, hearts were excised and processed for membrane isolation 24 hr after the last administration of the drug in order to avoid the acute effect of the last dose and to measure the chronic remanent effect of the drug. Myocardial amiodarone concentration at that time reached 6.9 ± 1.9 μg/g fresh weight.

DISCUSSION

The aim of this study was to test if amiodarone could affect, directly or indirectly, the binding of a specific radioligand to β-adrenergic receptor sites at the surface of the myocardial cell. Amiodarone has been shown to antagonize isoprenaline-induced tachycardia in the dog. The mechanism of inhibition has been characterized as non-competitive in that complete blockade of β-receptors could not be achieved even at high dosages [3]. Moreover, dose–response curves of the chronotropic effect of isoproterenol on isolated rabbit atria, recorded in the presence of various amiodarone concentrations, were characteristic of non-competitive inhibition [4]. In accordance with these data, our present results show that amiodarone does not compete with [^{125}I]cyanopindolol for binding to rat-heart β-adrenoceptors and thus clearly differs in its mechanism of action from classical β-blocking drugs. Our findings do not agree with recently published data suggesting strong competition between amiodarone and [^{3}H]dihydroalprenolol for specific binding to dog and rabbit microsomes [14].

Our data strongly suggest that amiodarone decreases the density of cardiac β-adrenergic receptors in rat-heart membranes, both *in vitro* and *in vivo* after acute or chronic treatment. We found no evidence of any change in receptor affinity for [^{125}I]CYP except after long-term administration where K_D values appeared slightly reduced. This decrease in the number of adrenergic receptors induced by amiodarone seems specific since 5'-nucleotidase activity was not affected by the drug, either *in vitro* or *in vivo*. Membrane bound 5'-nucleotidase is a specific marker of the sarcolemma

Table 2. Effect of amiodarone treatment on β-adrenergic receptor number and 5'-nucleotidase activity of rat-heart membranes

	B_{max} (fmoles/mg protein)	K_D (pM)	5'-Nucleotidase activity (μmoles/hr × mg protein)
Acute treatment			
Control (N = 12)	22.7 ± 0.6	98 ± 11	2.45 ± 0.12
Treated (N = 12)	19.1 ± 0.7	83 ± 10	2.46 ± 0.10
	$p < 0.001$	N.S.	N.S.
Chronic treatment			
Control (N = 12)	27.3 ± 0.9	100 ± 6	2.61 ± 0.13
Treated (N = 18)	23.0 ± 0.8	80 ± 4	2.44 ± 0.07
	$P < 0.001$	$P < 0.005$	N.S.

Data are the mean ± S.E.M. from N animals.

and the ratio of 5′-nucleotidase activity to β-adrenergic receptor number has been shown to be fairly constant in rat-heart membrane preparations of various degrees of purity [15].

In recent years, regulation of the adrenergic response through variations in the number of adrenergic receptors has received much attention. Besides up-and-down regulation through α- and β-blocking drugs and catecholamines themselves, changes in adrenergic receptors have been observed in several physiopathological states or after various kinds of pharmacological treatment (see [16] for a review). For instance, the cardiac β-adrenergic receptor number has been shown to vary according to the thyroid condition [17], in hypertension [15, 18], in congestive heart failure [19] and in myocardial ischemia [20]. Our results suggest that part of the β-antiadrenergic properties of amiodarone could be due to a decrease in β-receptor number at the surface of the myocardial cell. Whether or not this decrease in β-receptor number is transmitted to more distal events is under current investigation.

Interestingly, the amiodarone concentration required to induce *in vitro* a 50% decrease in β-adrenergic receptor number (IC_{50}) was consistent with the pD_2' value measured for the non-competitive inhibition of isoprenaline-induced tachycardia on isolated rabbit atria [4]. Moreover, decreases in β-adrenoceptor number in rat heats were observed after treatments which determined myocardial amiodarone levels of the same order of magnitude as those observed in humans under chronic therapy [21]. We thus make the hypothesis that the alteration in β-adrenoceptor number we observed in rat heart is relevant to the clinical situation.

The mechanism for the amiodarone-induced decrease in cardiac β-adrenoceptors in the rat is far from being obvious. We will consider three possible mechanisms. First, change in β-receptors observed *in vitro* may reflect a direct acction of amiodarone on the structure of the sarcolemmal membrane leading to alteration, solubilization or internalization of β-adrenoceptors. The amphiphilic nature of the amiodarone molecule would favour this hypothesis. The same mechanism of action would possibly operate *in vivo*. Second, the decrease in receptor number observed *in vivo* after chronic treatment could also result from increased proteolytic degradation and/or decreased synthesis of β-adrenergic receptors. Third, the change in β-receptor density may be due to a decrease in cardiac triiodothyronine (T_3) level. Indeed, it is known that amiodarone treatment induces a decrease in serum T_3 accompanied with an increase in serum T_4 and rT_3 [23, 24] and it has been claimed that the concentration of β-adrenoceptors in the heart is a function of the thyroid status, hypothyroidism inducing a decrease in β-receptors [17].

The present studies show that amiodarone treatment decreases the number of β-adrenergic receptors in rat-heart membranes, but additional studies will be required to identify the mechanism(s) responsible for this change in β-receptor number, and the physiological consequences and implications in this phenomenon.

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