*Biochemical Pharmacology*, Vol. 41, No. 6/7, pp. 1039–1044, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00


# REGULATION OF β-ADRENOCEPTORS BY THYROID HORMONE AND AMIODARONE IN RAT MYOCARDIAC CELLS IN CULTURE

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(*Received* 2 *August* 1990; *accepted* 29 *October* 1990)

**Abstract**—Hyperthyroidism is associated with elevation of heart cells sensitivity to catecholamines. We demonstrated that $T_3(10^{-8}\,M)$ increased (30%) the number of β-adrenoceptors in intact heart cells grown *in vitro* within 48 hr, without changing the affinity of the ligand [$^3$H]CGP-12177. The increase in β-adrenoceptors in $T_3$-treated myocytes was not associated with an increase in receptor-mediated cAMP production. Amiodarone, an antiarrhythmic drug, reduces the sensitivity of the heart to catecholamines. To investigate this effect, we analysed the influence of amiodarone on the level of β-adrenergic receptors. Ninety minute preincubation with amiodarone ($5 \times 10^{-5}\,M$) decreased the number of β-adrenoceptors (35–50%) in intact heart cells and in heart membranes, without affecting the dissociation constants ($K_d$). Amiodarone inhibited isoproterenol induced cAMP production. These results indicate that the mechanism of action of amiodarone on the heart seems to be a non-competitive inhibition of catecholamine receptors.

Many of the physiological and biochemical responses characteristic of hyperthyroidism are similar to those induced by adrenergic stimulation. It has been demonstrated that injection of rats with thyroxine ($T_4$) leads to an increase in β-adrenoceptor number in membranes derived from rat heart [1, 2]. Similar changes have been described in cultured heart cells [3, 4]. Thus, thyroid hormone (TH) regulation of β-adrenoceptor number may be a mechanism contributing to the hyper β-adrenergic state seen in hyperthyroidism.

Amiodarone is widely used for the treatment of cardiac arrhythmia and angina pectoris [5, 6]. In addition, amiodarone has been demonstrated to modulate TH-metabolism by reducing 5′-deiodination of ($T_4$) to triiodothyronine ($T_3$) and inducing a preferential production of reverse $T_3$ ($rT_3$) [7]. Venkatesh *et al.* [8] has shown that amiodarone caused a decrease in serum level of $T_3$ in adult rat. The changes induced by the drug closely resemble those of hypothyroidism [9]. In this state of hypothyroidism, the heart cells are less sensitive to catecholamines and a decrease in contractility is observed. These effects are probably mediated by a decrease in the number of β-adrenoceptors [1, 10]. In the present research, an attempt has been made to investigate the mechanism of action of $T_3$ and amiodarone in intact heart cells in culture and to further characterize the β-adrenoceptors following amiodarone treatment. We also support the finding that amiodarone antagonizes the effect of catecholamines through a non-competitive mechanism [11, 12].

## MATERIALS AND METHODS

*Preparation of cultures.* One to two-day-old rat hearts were removed under sterile conditions and washed three times in phosphate buffered saline (PBS) to remove the excess blood cells. The hearts were minced to small fragments and then gently agitated in proteolytic enzyme—RDB (Ness-Ziona, Israel) prepared from a fig tree extract. The RDB was diluted 1:50 in PBS, at 37° for a few cycles of 10 min each (as previously described [13, 14]). The supernatant suspensions containing dissociated cells, to which medium containing 10% horse serum (HS) (Biolab, Jerusalem, Israel) was added, were centrifuged at 150 *g* for 5 min. After centrifugation the supernatant was discarded and cells were resuspended in high glucose (5 mg/mL) Dulbecco's modified Eagle medium (DMEM) (Gibco, Uxbridge, U.K.) supplemented with 10% heat-inactivated HS and 2% chick embryo extract. The suspension of cells was diluted to $1.2 \times 10^6$ cells/mL and 1.5 mL was placed in 35 mm plastic culture dishes coated with collagen–gellatin. Cultures were incubated in a humidified 10% $CO_2$, 90% air at 37°. Confluent monolayers which exhibit spontaneous synchronous contractions, developed within 2–3 days in culture. The growth medium was replaced every 2–3 days.

*Hormonal and drug treatments.* Triiodothyronine ($T_3$) was dissolved in 0.05 N NaOH and applied to heart monolayer culture dishes to a final concentration of $10^{-8}\,M$ for 48 hr, or as specified. The level of thyroxine and $T_3$ in the serum was $1.5 \times 10^{-12}\,M$ and $3 \times 10^{-13}\,M$ respectively.

Amiodarone-HCl was dissolved in $H_2O$ at 60° to $10^{-3}\,M$, and applied to the cell cultures for 1–2 h before binding experiments. Protein determination was performed according to the Lowry method, using bovine serum albumin as a standard [15]. Creatine kinase (CK) activity was measured using CK kit (Biotrol, France) and the NADH produced by the enzyme was measured spectrophotometrically as previously described [16].

*Ligand binding.* β-Adrenoceptor binding was measured as previously described [17]. Briefly, intact cells were incubated at room temperature (22–25°) for 45 min with various concentrations of the β-adrenergic antagonist [$^3$H]CGP-12177 ((−)[$^3$H](4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidazol-2-one)) in PBS, pH 7.4. Incubation was stopped by rinsing the cells seven times with cold (4–10°) PBS. The cells were solubilized with 0.3 mL Triton X-100 (1%) and radioactivity was determined in scintillation counter. Non-specific binding of [$^3$H]CGP-12177 was defined as the amount of radioactivity remaining after incubation with L-alprenolol ($10^{-4}$ M). Specific [$^3$H]CGP-12177 binding was calculated as the total minus the non-specific binding (less than 20%).

For inhibition experiments, cells were incubated with amiodarone for 1–2 hr at 37° in the growth medium. The medium was then replaced with PBS in the presence of amiodarone and the binding was performed with [$^3$H]CGP-12177 (3 nM) at room temperature for 45 min (unless otherwise indicated).

For membrane binding experiments, cells were scraped off the dish (with a rubber policeman) into 1 mL cold PBS and homogenized for 5 sec. The binding experiment was performed in the total volume of 1 mL homogenates and terminated by filtering through Whatman GF/C filters, washing five times with 1 mL cold PBS and drying the filters before counting the radioactivity.

*cAMP accumulation.* Intracellular cAMP levels were measured in the heart cells. Assays were carried out at least in duplicate for each treatment. The cells were preincubated with the appropriate treatment at 37° in growth medium and 5 mM theophylline. After the indicated treatment the cells were rinsed twice with cold PBS and scraped off the dish (with a rubber policeman) in 500 μL ethanol 95% [18]. The cells were homogenized for 5 sec and centrifuged at 2000 *g* at 0° for 5 min. Pellets were used for protein determination. Supernatants were centrifuged in a speed vacuum centrifuge for 90 min to evaporate the ethanol. One hundred microlitres of 0.05 M Tris–HCl pH 7.5 with EDTA 4 mM were added (to inhibit the phosphodiesterases) to the pellets and cAMP assayed according to the Amersham kit protocol.

All drugs and chemicals used were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [$^3$H]CGP-12177, sp. act. 48 Ci/mmol and [$^3$H]cAMP, sp. act. 180 pmol ~5 μCi were purchased from Amersham (Little Chalfont, Bucks, U.K.).

## RESULTS

### *Characterization of β-adrenoceptor on intact heart cells*

Myocardial cells, 4–5 days old, were treated with 10 nM $T_3$ for 48 hr. A 30% increase of [$^3$H]CGP-12177 binding was observed following $T_3$ treatment, in comparison to control. Scatchard analysis of these data indicates that the maximum binding sites was 35 fmol/dish (58 fmol/mg protein) in untreated cells and 58 fmol/dish (86 fmol/mg protein) in $T_3$ treated cells (Fig. 1). The number of binding sites per cell in the control was 24,000 ± 2000 in comparison to 34,000 ± 2600 in $T_3$ treated cells. The $K_d$ for [$^3$H]CGP-12177 binding was 0.6 ± 0.1 nM and 0.7 ± 0.1 nM for the control group and $T_3$ treated group respectively (insignificant differences).

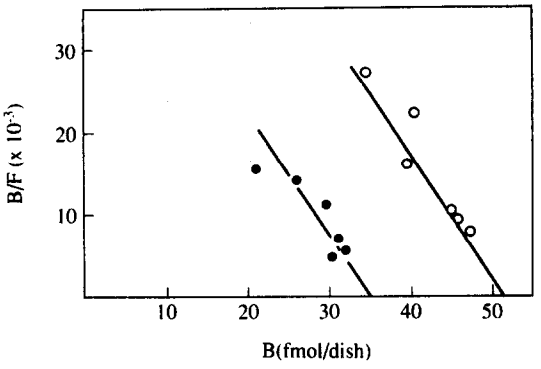


Fig. 1. Scatchard plot of specific binding of [$^3$H]CGP-12177 to cultured rat heart cells from control and $T_3$-treated cultures. Five-day-old rat cardiomyocytes were treated with $T_3$($10^{-8}$ M) 48 hr, and binding was carried out as described in Materials and Methods. Specific binding was defined as the [$^3$H]CGP-12177 binding displaceable by $10^{-4}$ M L-alprenolol. Data points are the means of triplicate determinations. The $K_d$ for [$^3$H]CGP-12177 from the control (closed symbols) and $T_3$-treated cells (open symbols) were 0.6 and 0.7 nM respectively. The maximal binding capacity was 35 fmol/dish for the control and 58 fmol/dish for the $T_3$ treated cells. (The average amount of protein per dish was 0.6 mg).

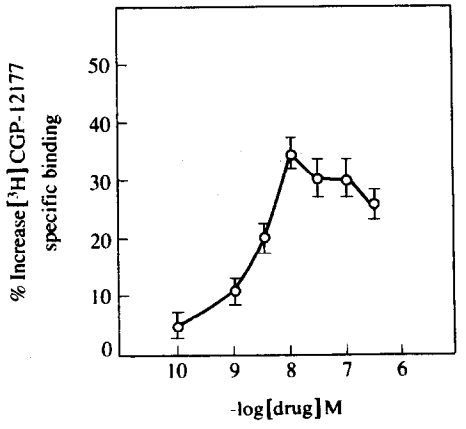


Fig. 2. Dose effect of $T_3$ on [$^3$H]CGP-12177 binding. Myocardial cells (3-day-old) were treated for 48 hr with $T_3$ at different concentrations. Specific binding was then determined as described in Materials and Methods. Each point represents means (±SE) of three different experiments.

The dose effect of $T_3$ on myocardial cells β-adrenoceptors is shown in Fig. 2. The maximum increase of β-adrenoceptors was achieved at $10^{-8}$ M of $T_3$. Higher concentrations of $T_3$ ($10^{-6}$ M) caused toxic effects, as revealed by a decrease in CK activity in the cell homogenates (data not shown). To determine the age dependence of β-adrenoceptors appearance in $T_3$-treated and untreated myocytes,

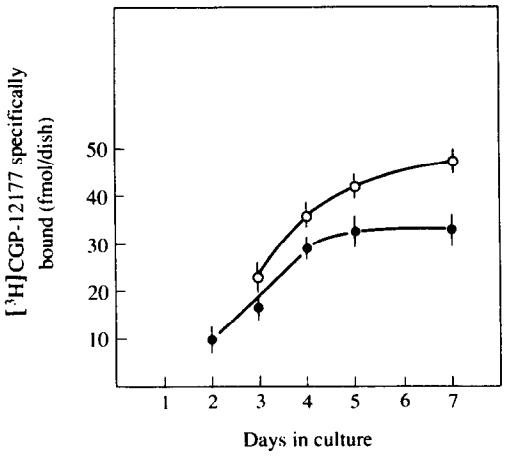


Fig. 3. Age dependent specific binding of [³H]CGP-12177 to heart cells from control and $T_3$-treated cultures. Every day different cells were treated with $T_3(10^{-8}$ M) for 48 hr and binding experiment was performed as described in Materials and Methods. Each point is the mean (±SE) of three separate experiments performed in triplicate. Control: closed symbols. Thyroid hormone treated: open symbols.

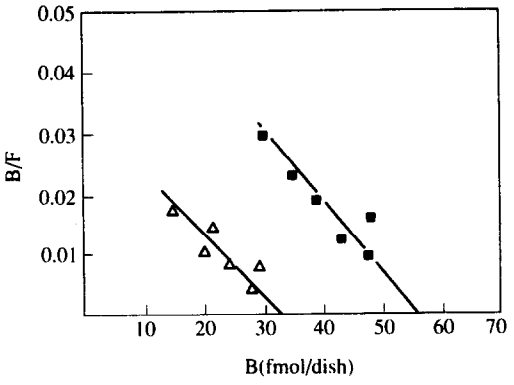


Fig. 4. Effect of amiodarone on β-adrenoceptors. Heart cells (5-day-old) were treated with amiodarone $5 \times 10^{-5}$ M for 90 min, and binding of [³H]CGP-12177 was performed. Scatchard analysis of the data shows that the $K_d$ for [³H]CGP-12177 from control (closed symbols) and amiodarone-treated cells (open symbols) are 0.85 and 0.9 nM respectively. The $B_{max}$ of control and amiodarone treated cell are 56 fmol/dish and 33 fmol/dish respectively (amount of protein/dish = 1.1 mg). Data points represent means of duplicate determinations from three experiments.

Table 1. cAMP accumulation in myocardial cells following $T_3$ treatment

| | cAMP accumulation (pmol/mg protein) Treatment 48 hr |
|---|---|
| Control | 17.0 |
| $T_3$ | 19.0 |
| Isoproterenol | 33.0 |
| $T_3$ + Isoproterenol | 34.0 |

Cells were treated with $T_3$ ($10^{-8}$ M) for 48 hr. cAMP level in $T_3$-treated cells and untreated cells were measured with or without 30 min Isoproterenol-induced cAMP accumulation, as described in Materials and Methods. These data represent means of duplicate determinations of one representative experiment. (This experiment was repeated three times with consistent results.)

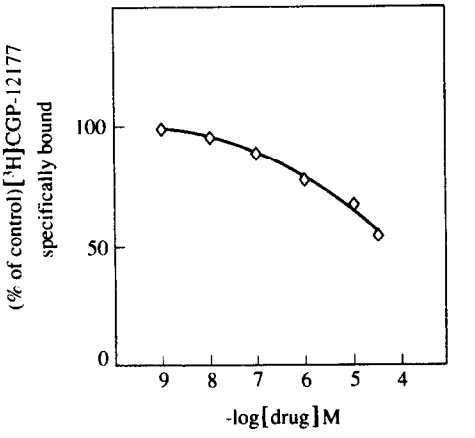


Fig. 5. Dose–effect of amiodarone on [³H]CGP-12177 binding. Myocardial cells (5-day-old) were preincubated for 90 min with amiodarone at different concentrations. Specific binding was determined as described in Materials and Methods. Each point represents means of three different experiments.

the cells received $T_3$ for 48 hr at various times during their development prior to binding experiments. There was a linear increase in the level of β-adrenoceptors during the first 4 days in culture, then the level of the receptors reached a plateau the level of β-adrenoceptor was higher than that of control. The maximum effect of $T_3$ on the myocardial cells in elevating the β-adrenoceptor number was achieved when the cells reached this plateau (5–7-day-old cultures) (Fig. 3).

In order to study whether the new β-adrenoceptors were coupled with adenylate cyclase activity, we measured cAMP accumulation in $T_3$-treated cells. Table 1 shows that $T_3$ treatment for 48 hr did not alter basal or isoproterenol ($10^{-4}$ M) stimulated a cAMP production compared to control cells (Table 1).

*Inhibition of* [³H]*CGP-12177 binding by amiodarone*

Ninety minute preincubation with amiodarone resulted in 40% decrease in the number of [³H]CGP-12177 binding sites. In amiodarone-treated cells the number of binding sites was 33 ± 2.5 fmol/dish (30 fmol/mg protein) and in untreated cells the number of binding sites was 56 fmol/dish (50 fmol/mg protein). The equilibrium dissociation constant for the interaction of the ligand with its binding sites is 0.9 ± 0.09 nM and 0.85 ± 0.1 nM in amiodarone and in control respectively (Fig. 4). The dose effect of amiodarone on the level of β-adrenoceptors shows that up to $10^{-8}$ M, amiodarone did not interfere with [³H]CGP-12177 binding (Fig. 5). At higher

Table 2. Inhibition effect of amiodarone on [$^3$H]CGP-12177 binding

| | Control | Amiodarone | % Inhibition |
|---|---|---|---|
| Bound in cells (fmol/mg protein) | 50 ± 2.5 | 32 ± 3.1 | 35 |
| Bound in membrane (fmol/mg protein) | 40 ± 1.5 | 20 ± 2.2 | 50 |
| CK-content (mUnits/mg protein) | 800 ± 50 | 850 ± 30 | |

Intact cells and membranes were incubated 90 min with $5 \times 10^{-5}$ M amiodarone at 4° and the binding experiment and CK-content was performed as described in Materials and Methods. These data are the mean ± SE obtained from one representative experiment and performed in triplicate.

Table 3. Inhibition of isoproterenol-induced cAMP accumulation in heart cells by amiodarone

| | cAMP accumulation (pmol/mg protein) | |
|---|---|---|
| | Treatment 30 min | Treatment 90 min |
| Control | 11.7 | 12.0 |
| Isoproterenol | 23.5 | 27.0 |
| Amiodarone | 11.8 | 13.0 |
| Isoproterenol + amiodarone | 20.0 | 12.9 |

Cells were treated for 30 or 90 min with isoproterenol ($10^{-4}$ M), amiodarone ($5 \times 10^{-5}$ M) or isoproterenol + amiodarone. cAMP production was measured as described in Materials and Methods. These results represent means of duplicate determinations of one representative experiment. (This experiment was repeated three times with similar results.)

concentrations, however, amiodarone decreased radioligand binding in a dose-dependent manner (at higher concentration than $5 \times 10^{-5}$ M, amiodarone precipitated).

To investigate whether amiodarone provokes internalization of β-adrenoceptors, the inhibition of [$^3$H]CGP-12177 binding by amiodarone was tested in intact cells and in cardiac membranes. In intact cells preincubated with amiodarone at 4° and in cardiac membranes the inhibition of [$^3$H]CGP-12177 binding was 35 and 50% respectively (Table 2). The low temperature did not abolish the inhibition of [$^3$H]CGP-12177 binding by amiodarone, making the possibility of internalization by the drug unlikely. Creatine kinase activity was also determined following amiodarone treatment to eliminate toxicity or cell-loss as a result of drug treatment (Table 2).

To investigate whether amiodarone interfered with the isoproterenol induced cAMP accumulation, intact cardiac cells were incubated for 30 min or 90 min with isoproterenol together with amiodarone, and the level of cAMP was measured. Amiodarone did not change the basal level of cAMP in comparison to the control untreated cells but inhibited isoproterenol induced cAMP production (Table 3). This inhibition was much more pronounced following 90 min pretreatment with isoproterenol and amiodarone, probably because the isoproterenol affinity for the receptors is much higher.

## DISCUSSION

It has become apparent in the past several years that β-adrenoceptors may be dynamically regulated by a wide variety of physiological and pathophysiological influences [19]. Several studies have tried to demonstrate a direct effect of thyroid hormone on β-adrenoceptors. The *in vitro* model of the neonatal rat heart cell in culture enabled the possibility to address this question directly. In cultured cells, indirect effects of thyroid hormone on cardiac receptors, by actions via other organ systems, are eliminated.

Cultured cardiac cells exposed for 48 hr to triiodothyronine demonstrated a 30% increase in the number of β-adrenoceptors. The increase in β-receptor number is not accompanied by a significant change in binding affinity, nor by a change in total protein content. This increase in the β-adrenoceptors is probably a result of increased receptor synthesis, since the protein synthesis inhibitor, cycloheximide (5 μg/mL), prevented $T_3$ stimulation (data not shown). In contrast to other studies showing that elevated TH levels tend to cause hypertrophy [20, 21], total protein content and CK activity [13, 22] were not significantly changed in our cultured heart cells following 48 hr $T_3$ treatment.

The number of [$^3$H]CGP-binding sites reported by Kim *et al.* [4] is lower than found by us, 28 vs 58 fmol/mg protein respectively. This difference can be explained by the existence of a species difference between the two system models. Whereas Kim *et al.* [4] worked on cardiomyocytes obtained from chick embryos, we work on cultures from newborn rats. In spite of these differences, the induction of β-adrenoceptors by $T_3$ is similar (40%). Kupfer *et al.* [23] also measured β-adrenoceptors using [$^{125}$I]ICYP as radioligand. They also worked on rat myocardial cells and obtained 12,000 sites per cell, which is lower than our number: 24,000 sites/cell. The differences between our results and the above mentioned authors is probably due to the fact that they used isolated heart membranes, whereas we have measured the β-adrenoceptors in intact cardiomyocytes.

We show in Fig. 3 that the influence of the hormone on the development of the receptors is largest at plateau level (5 days old). It might be that the appearance of the binding sites for $T_3$ requires the differentiation of the cardiomyocytes *in vitro*.

Similar results were obtained in our laboratory showing that TH caused a transition of CK isozymes in the cardiomyocytes in cultures in an age-dependent manner [13].

Kim *et al.* [4] reported that exposure to thyroid hormone increased not only β-receptors level as previously mentioned, but also the number of slow $Ca^{2+}$ channels, in the sarcolemmal membrane of cultured chick heart cells. They showed that the increased myocardial contractility observed in the hyperthyroid heart appears to be partially due to increased number of slow $Ca^{2+}$ channels. This increase in contractility can explain the increase in β-adrenoceptors.

Although $T_3$ increased β-adrenoceptors, there was no corresponding increase in isoproterenol induced cAMP production suggesting that the receptors are not functional. One of the possibilities is that this process is time dependent and the receptors are not yet coupled with adenylate cyclase activity. These data are in contrast to the results obtained by Tsai and Chen [3] who demonstrated that $T_3$ increased β-adrenoceptor level, and agonist-induced cAMP production. These results are, however, surprising since they reported that adrenaline provoked cAMP accumulation for 48 hr. Such duration of agonist treatment is generally considered to cause down-regulation of β-adrenoceptors [24].

Amiodarone has been shown to antagonise catecholamines induced cardiovascular effects. The mechanism of inhibition of adrenoceptors has been characterised as non-competitive type [12]. Our data suggest that amiodarone decreases the density of cardiac β-adrenoceptors in rat heart intact cells following acute (90 min) or chronic (48 hr) treatment. No evidence has been found for any significant change in receptor affinity for [$^3$H]CGP-12177. This decrease in the number of β-adrenoceptors induced by amiodarone seems to be specific, since creatine kinase activity was not affected by the drug, nor by TH [22]. Similar results were obtained in cardiac membranes using [$^{125}$I]ICYP to label β-adrenoceptors [23].

The amiodarone concentration required to induce 50% decrease in β-adrenoceptor number ($IC_{50}$) was consistent with the $PD'_2$ value measured for the non-competitive inhibition of isoprenaline-induced tachycardia on isolated rabbit atria [11]. Thus, we confirm the hypothesis proposed by others [12] that the alteration in β-adrenoceptor number in rat heart following amiodarone treatment is relevant to the clinical situation. The mechanism for amiodarone-induced decrease in cardiac β-adrenoceptors is unclear. Since similar decrease in the receptor density was observed in intact cardiomyocytes treated at 4° and in membrane prepared from these cells, the possibility that amiodarone induces internalization process seems unlikely. Additional studies are required to identify the mechanism responsible for the decrease in β-adrenoceptor number.

It has been discussed that amiodarone antagonizes hormone-dependent adenylate cyclase stimulation in heart plasma membrane without any effect on the basal activity of adenylate cyclase [12]. Similar results were obtained by us in intact cells treated with amiodarone. Further studies are required to underly the mechanism of anti-adrenergic action of amiodarone as a non-competitive inhibitor of the receptors.

*Acknowledgements*—This work was partially supported by funds from the Otto Meyerhoff Drug Receptor Center and from the Health Science Research Center at Bar-Ilan University.

Thanks to A. Isaac and T. Zinman for their valuable technical assistance and to C. Scop for typing the manuscript.

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