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EFFECTS OF CALCIUM AND CALCIUM-CHANNEL BLOCKER METHOXYVERAPAMIL ON THE β-ADRENOCEPTORS IN MYOCARDIAL CELLS *IN VITRO*

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Abstract—The possible relationship between methoxyverapamil (D600) as a calcium-channel blocker and the β-adrenoceptors was investigated on heart cells grown in culture, using [^{3}H]CGP-12177 as a radioligand. Treatment with D600 (20 μg/mL) for 24 hr caused a decrease of 30% in the [^{3}H]CGP-12177 binding sites. Scatchard analysis showed that the B_{max} is similar in control and D600-treated cells, but the K_d in D600-treated cells increases. The effect of D600 on the isoproterenol-induced adenylate cyclase activation was examined and it was found that the D600 prevented the increase in cAMP obtained by isoproterenol treatment. These results indicate that the action of D600 on the β-adrenoceptors is a competitive inhibition of the [^{3}H]CGP-12177 binding sites. We investigated the effect of Ca^{2+} in the growth medium on the level of β-adrenoceptors. Heart cells grown for 24 hr in Ca^{2+}-free medium showed a decrease of 36% in the [^{3}H]CGP-12177 binding sites without changing the dissociation constant. This decrease is probably a result of reduction in synthesis of the receptors. The level of receptors returned to control values following replenishment with normal growth medium. These results show that calcium is essential for the development of the β-adrenoceptors in heart cells *in vitro*.

Calcium-channel blockers, such as verapamil or methoxyverapamil (D600†), are useful drugs in the treatment of several cardiovascular disorders, including cardiac arrhythmias. Recent studies indicate that antagonism of Ca^{2+}-channels may not be the only mechanism whereby these drugs alter myocardial function. Several reports have shown that verapamil can alter membrane receptors such as muscarinic acetylcholine receptors, and α-adrenergic and β-adrenergic receptors in different tissues, mainly in rat brain membranes [1–5]. Intact cells are an appropriate system to study the mechanism mediating the action of Ca^{2+}-channel blockers on the cardiovascular system.

Considering the importance of Ca^{2+} in the process of heart muscle contraction, it is of interest to understand the role of Ca^{2+} ions in the regulation of the level of β-adrenoceptors. Birnbaum *et al.* [6] reported that there is a correlation between the number of AChR in skeletal muscle grown in culture and Ca^{2+} concentration in the medium $[Ca^{2+}]_o$. They showed that high $[Ca^{2+}]_o$ causes an increase in AChR synthesis. It was suggested [6] that the expression of AChR gene is stimulated specifically by high $[Ca^{2+}]_o$. In contrast, low $[Ca^{2+}]_o$ has been reported to decrease the rate of synthesis of these receptors in myotubes grown in cell culture [6].

Gengo *et al.* [7] reported that chronic administration of nifedipine (Ca^{2+}-channel blocker) produced down-regulation of cardiac and neural Ca^{2+}-channels and a similar down-regulation of β-adrenoceptors. However, these results have not been confirmed for all Ca^{2+}-channel blockers [8]. On the contrary, Hedberg *et al.* [9] observed a 46–65% increase in the number of β-receptors in human atria treated with calcium antagonist and similar results were obtained in cultured cardiac myocytes isolated from neonatal rat [10]. Thus, the role of calcium in the modulation of membrane proteins deserves further characterization. In this study we examined the effect of $[Ca^{2+}]_o$ and Ca^{2+}-channel blocker D600 on the level of β-adrenoceptors, and the mechanism by which they exert their effects.

MATERIALS AND METHODS

Preparation of heart cell cultures. Rat hearts (1–2-day-old) were removed under sterile conditions and washed three times in PBS to remove the excess blood cells. The hearts were minced into 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 described previously [11–13]. The supernatant suspensions containing dissociated cells to which medium containing 10% horse serum (Biolab, Jerusalem, Israel) was added, were centrifuged at 150 *g* for 5 min. After centrifugation the supernatant phase was discarded and cells were resuspended in high glucose (5 mg/mL) Dulbecco's Modified Eagle Medium (Gibco, Uxbridge, U.K.) supplemented

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† Abbreviations: AChR, acetylcholine receptors; [^{3}H]-CGP-12177, (−)[^{3}H](4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidazol-2-one); CK, creatine kinase; D600, methoxyverapamil; PBS, phosphate-buffered saline.

with 10% heat-inactivated horse serum and 2% chick embryo extract. The suspension of cells was diluted to 1.2×10^6 cells/mL and 1.5 mL were placed in 35 mm collagen-coated plastic culture dishes. Cultures were incubated in a humidified 10% CO_2, 90% air at 37°. Confluent monolayers, which exhibit spontaneous contractions, developed in the cultures within 2–3 days. The growth medium was replaced every 3–4 days.

Hormonal and drug treatments. D600 was dissolved in water and applied to heart monolayer culture dishes at a final concentration of 20 μg/mL, or as specified.

For the Ca^{2+}-deficient medium treatment, the cells were washed twice with PBS (calcium- and magnesium-free) and incubated for 24 hr in Ca^{2+}-deficient medium without serum or embryo extract. Protein determination was performed according to Lowry *et al.* [14] using bovine serum albumin as a standard. CK activity was measured using CK kit (Biotrol, France) and the NADH produced by the enzyme was measured spectrophotometrically as described previously [15].

Ligand binding. Intact cells were incubated at room temperature (22–25°) for 45 min, with various concentrations of the β-adrenergic antagonist [^{3}H]-CGP-12177, in PBS, pH 7.4 as described previously [13]. 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 by scintillation counting. 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 radioactivity bound minus the non-specific binding (less than 20%).

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 using a total volume of 1 mL of homogenates and was terminated by filtering them through Whatman GF/C filters, washing them 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 twice for each experiment. The cells were preincubated with the appropriate treatment at 37° in the 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% [16]. The cells were homogenized for 5 sec and centrifuged at 2000 *g* at 0° for 5 min. Pellets were used for protein determination. The supernatant was centrifuged in a speed vacuum centrifuge for 90 min to evaporate ethanol. To the obtained pellet which contained cAMP, 100 μL 0.05 M Tris–HCl pH 7.5 with EDTA 4 mM was added (EDTA inhibits the phosphodiesterases). cAMP was 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. 53 Ci/mmol, and [^{3}H]cAMP, sp. act. ~ 5 μCi/180 pmol were purchased from Amersham International (Amersham U.K.).

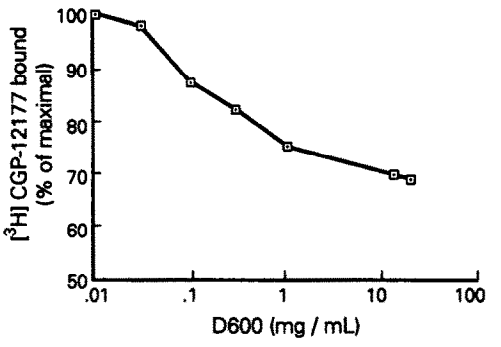

Fig. 1. Inhibition of [^{3}H]CGP-12177 binding by D600. Myocardiac cells (5-day-old) were treated with D600 at various concentrations and [^{3}H]CGP-12177 binding was measured 24 hr later. The points are means of three experiments performed in duplicate.

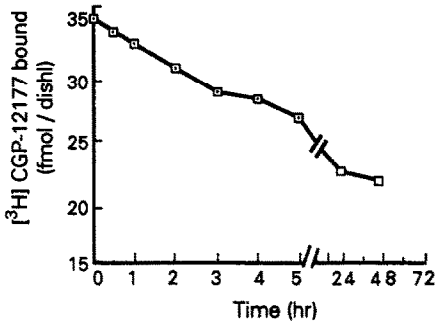

Fig. 2. Time-course of D600 on [^{3}H]CGP-12177 binding. Myocardiac cells were incubated with D600 (20 μg/mL) for various intervals of time and [^{3}H]CGP-12177 binding was measured as described in Materials and Methods. The points are means of three experiments performed in duplicate.

RESULTS

The influence of a calcium-channel blocker on the β-adrenoceptors

Myocardial cells grown in culture were treated with various concentrations of D600 for 24 hr and the level of β-adrenoceptors was then measured on intact cells. The maximal inhibition of [^{3}H]CGP-12177 binding (30%) occurred between 13 and 20 μg/mL (Fig. 1). It is worth mentioning that the spontaneous contractions of the cells were inhibited at 0.3 μg/mL, however, at this concentration no significant inhibition of [^{3}H]CGP-12177 binding was obtained (Fig. 1).

The time-course of D600 (20 μg/mL) on [^{3}H]CGP-12177 binding shows that the effect of D600 on the level of [^{3}H]CGP-12177 binding sites increased as a function of time. The maximum effect was achieved after 24 hr. No further change was observed between 24 and 48 hr (Fig. 2). CK activity was determined following D600 treatment to eliminate toxicity or

Table 1. Inhibition of isoproterenol-induced cAMP production by D600

	cAMP accumulation (pmol/mg protein)
Control	10.6 ± 0.8
D600	10.0 ± 0.5
Isoproterenol	33.1 ± 1.5
D600 + isoproterenol	18.1 ± 1.1

Six-day-old myocardiac cells were treated for 24 hr with D600 (20 μg/mL) and cAMP production was measured in the cells after 30 min of isoproterenol treatment. The results are from one representative experiment performed in triplicate.

cell loss as a result of drug treatment and showed no significant change. CK activity in control cells was 1.7 ± 0.1 U/mg protein and in D600-treated cells was 1.75 ± 0.08 U/mg protein. (No significant changes were detected following 24 or 48 hr of D600 treatment.)

The relationship between the radioligand concentrations and the number of specific binding sites in D600-treated and control cells was analysed. Specific binding reached a plateau at a concentration of 3 nM of [^{3}H]CGP-12177 in both groups. At this concentration of the ligand, D600 inhibited ligand binding by 30 ± 2.6%. Scatchard analysis of these results (Fig. 3) shows that D600 did not change the number of β-receptors; B_{max} is 36.5 fmol/dish (45.6 fmol/mg protein). However, D600 decreased the affinity of the ligand to the receptors. The dissociation constant in D600-treated cells increased in comparison to control, untreated myocardial cells from 1.1 ± 0.3 to 4.2 ± 0.4 nM.

To study further the relationship between D600 and β-adrenoceptors, the involvement of D600 in isoproterenol-stimulated adenylate cyclase activity was examined. Myocardial cells were treated with D600 for 24 hr and the isoproterenol-induced cAMP accumulation was measured. Table 1 shows that treatment with isoproterenol, 10^{-4} M, for 30 min increased cAMP level in control cells as reported previously [15]. D600 did not affect the basal level of cAMP. However, D600 reduced the agonist-induced cAMP production by 64%, as expected according to the results obtained using the radioligand binding technique.

The effect of calcium on the β-adrenoceptors

To investigate the effect of calcium in the medium on the binding properties of the β-adrenoceptors, 5-day-old myocardial cells were transferred into a Ca^{2+}-free medium for 24 hr. Scatchard analysis of [^{3}H]CGP-12177 binding shows that a Ca^{2+}-deficient medium reduces the level of the receptors by 36% from 43.2 ± 1.8 fmol/dish in the control to 27.3 ± 1.5 fmol/dish in Ca^{2+}-deficient medium (Fig. 4). The calculated K_d for the control cells was 1.9 nM and for the Ca^{2+}-deficient medium cells was 1.6 nM. This difference was not statistically significant. The decrease in the level of β-adrenoceptors was specific since other muscle proteins such as CK were unaltered (Table 2). When the cells were kept in a

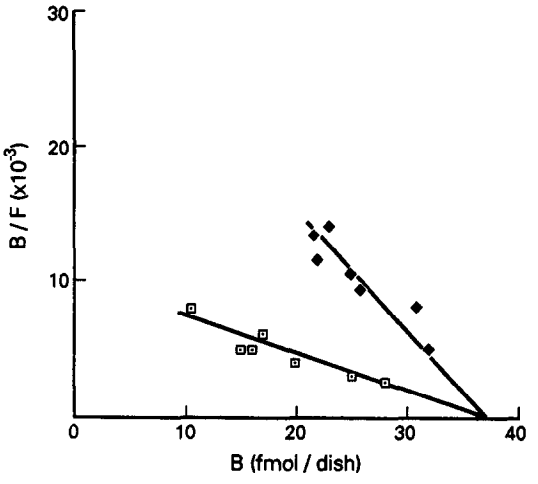

Fig. 3. Effect of D600 on β-adrenergic receptors of heart muscle cells. Five-day-old myocytes were treated with D600 (20 μg/mL) for 24 hr and binding was performed for 30 min on the control cells and on the D600-treated cells. Scatchard plot of specific binding [^{3}H]CGP-12177 to rat heart cells shows that the B_{max} for the two groups of cells is 36.5 ± 2.2 fmol/dish. The K_d for control cells (◆) is 1.1 ± 0.3 nM and for D600-treated cells (⊡) is 4.2 ± 0.4 nM (0.6 mg protein/dish).

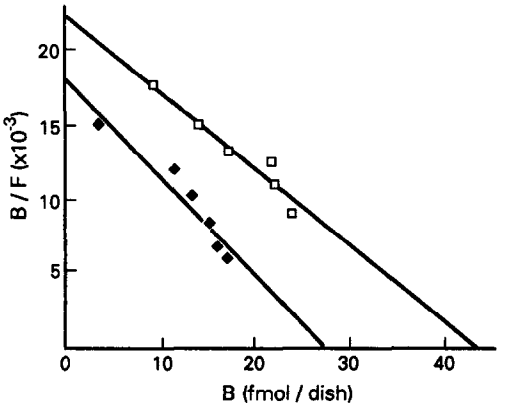

Fig. 4. Effect of calcium on β-adrenoceptors. Scatchard plot of specific binding of [^{3}H]CGP-12177 on myocardial cells. Binding was carried out as described in Materials and Methods. Heart cells (4–5-day-old) were incubated for 24 hr in the absence of calcium and binding experiment was performed for 45 min. The K_d values for [^{3}H]CGP-12177 from control (◆) and treated (□) cells are 1.9 and 1.6 nM, respectively. Data points represent means of duplicate determinations from three experiments.

Ca^{2+}-deficient medium (for 24 hr) and then returned to a normal growth medium (1.8 mM Ca^{2+}), the level of β-adrenoceptor binding sites returned to almost control level after 24 or 48 hr (Table 2). To clarify the mechanism of the decrease in receptors in a Ca^{2+}-deficient medium, the cells were treated with cycloheximide (3 μg/mL) and the half-life of the receptors was measured. No significant difference

Table 2. Effect of Ca^{2+}-deficient medium on the β-adrenoceptors

	[^{3}H]CGP-12177 bound (% of maximal)	CK content (U/mg protein)
Control	100	1.80 + 0.08
Ca^{2+}-deficient medium (24 hr)	65	1.75 ± 0.1
Ca^{2+} replenishment (24 hr)	82	1.90 ± 0.09
Ca^{2+} replenishment (48 hr)	90	1.85 ± 0.05

Six-day-old myocardiac cells were incubated for 24 hr in Ca^{2+}-deficient medium and then growth medium was replenished for the indicated times. In each treatment [^{3}H]-CGP-12177 bound and CK activity were measured as indicated in Materials and Methods. The data represent the results of one representative experiment performed in duplicate. (There are no statistically significant differences in the CK data.)

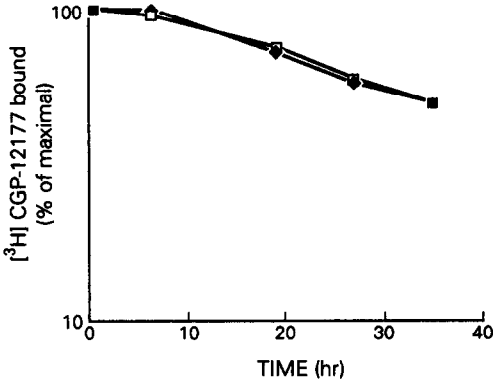

Fig. 5. The rate of degradation of the β-adrenoceptors. Five-day-old myocardiac cells were treated with cycloheximide (3 μg/mL) and binding experiment was performed at the time intervals indicated in the graph. The half-life of the two groups was found to be 35 hr. (□: control; ◆: Ca^{2+}-deficient medium). The data are means of triplicate determinations from three experiments.

was observed in the rate of degradation between the two groups of cells. The half-life was found to be 35 hr (Fig. 5). Thus, the reduction obtained in the level of β-adrenoceptors in a Ca^{2+}-depleted medium is probably a result of a decrease in the rate of synthesis of the receptors.

DISCUSSION

Calcium-channel blockers have clinical effects on the heart but it is not clear whether their mechanism of action is through the Ca^{2+} fluxes only. Many investigators have studied the capacity of verapamil or its methoxy-derivative, D600, to alter several types of membrane receptor [1–5]. Karliner *et al.* [17], working on rat myocardium membrane, showed that verapamil changed the affinity of the radioligand for the α-adrenergic and muscarinic receptors, without changing the affinity for the radioligand to the β-adrenoceptors. In contrast to these results, Feldman *et al.* [18] have reported that the addition of verapamil to broken lymphocyte cells results in an increase in the affinity constant of [^{125}I]ICYP (β-blocker) with no change in the maximal number of β-adrenergic receptors, which is in accordance with our work.

Our results show that D600 inhibited CGP-12177 binding sites in intact myocardial cell in a dose- and time-dependent manner (Figs 1 and 2). We noticed that at a very low concentration (0.3 μg/mL), D600 inhibited spontaneous contractions of the myocytes without altering the binding capacity of [^{3}H]CGP-12177. These results suggest that there is no correlation between the phenomenon of spontaneous contractions and the properties of β-adrenoceptors. To eliminate the possible toxic effect of D600, CK activity was determined in the myocardial cells. CK activity measured in D600-treated cells shows that drug treatment did not damage the cells.

Scatchard analysis of [^{3}H]CGP-12177 binding (Fig. 3) shows that D600 decreased the affinity of the radioligand to the receptor without changing the number of β-adrenoceptors. D600 appears to be a competitive inhibitor for binding of [^{3}H]CGP-12177. However, high concentrations of this Ca^{2+}-channel blocker are needed to block the CGP-12177 binding sites in comparison to a classical β-antagonist. The mechanism by which D600 exerts its inhibition is probably a direct interaction either with β-receptors or with membrane components in close proximity to the receptor binding site. Similar results were reported by Karliner *et al.* [17] on the effect of verapamil on the α-adrenergic and muscarinic acetylcholine receptors.

To further characterize the interaction of D600 with the β-adrenoceptors we studied its inhibition of isoproterenol-induced cAMP production. It is unlikely that calcium antagonists have β-adrenergic agonist effects because D600 did not induce cAMP generation even at the high concentration of 0.1 mM [19–22]. Isoproterenol induced cAMP production as reported previously [13]. In the present study we showed that D600 interferes with the isoproterenol-induced increase in the basal level of cAMP. These results contradict the findings of Yonemochi *et al.* [10] who observed a 40–50% increase in the number of β-adrenoceptors in cultured cardiac myocytes treated with calcium antagonists. Such elevation of the β-receptors would show increased expression of isoproterenol-induced cAMP production, which was not found in our study. Thus, our finding that D600 inhibited both [^{3}H]CGP-12177 binding sites and

isoproterenol-induced cAMP generation is consistent with the observations of Feldman *et al.* [18] on broken lymphocytes. The current results emphasize that when Ca^{2+}-channel blockers are used therapeutically, their effects should not be attributed exclusively to channel blocking.

In this report, we have shown that the level of β-adrenoceptors was reduced when the cells were grown in Ca^{2+}-deficient medium (Fig. 4). This reduction could be explained by deducing that lack of calcium damaged the cells. However, measurement of CK activity did not justify such a possibility: in the absence of calcium, the activity of CK in both groups was similar. Thus, the reduction of the level of β-adrenoceptors is specific. To study the mechanism of this reduction at the receptor level, we analysed the half-life of the β-adrenoceptors by using a protein synthesis inhibitor, cycloheximide. It was found that the half-life of β-adrenoceptors in Ca^{2+}-deficient medium is the same as that of the control cells. These data indicate that the decrease in β-adrenoceptors is a result of inhibition of receptor synthesis. To study whether this process is reversible Ca^{2+} was restored to the medium. However, calcium repletion creates a problem called "calcium paradox" which is implicated in the onset of cellular damage [22]. To overcome this problem, we kept the cells in Ca^{2+}-deficient medium for only 24 hr before Ca^{2+} was restored. Under these conditions, CK activity was not affected which indicated that the calcium paradox is not relevant to the embryonic cardiac cells during this incubation period.

In conclusion, calcium was found to be essential for the synthesis of β-adrenoceptors and the reason D600 did not cause the same reduction in the number of receptors as in Ca^{2+}-deficient medium is probably because the drug does not completely block Ca^{2+} entry into the cells due to the existence of D600-insensitive channels, as suggested previously [23].

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