Effects of Calcium Antagonists on \( \beta \)-Receptors of Cultured Cardiac Myocytes Isolated From Neonatal Rat Ventricle

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The effects of calcium antagonists (verapamil, diltiazem, and nicardipine) on \( \beta \)-adrenergic receptors of cultured cardiac myocytes isolated from neonatal rat ventricle were studied with the hydrophilic ligand \([\text{H}]\)CGP-12177, which identifies cell surface-bound \( \beta \)-receptors. The three calcium antagonists suppressed spontaneous beating of the myocytes, increased the number of \( \beta \)-receptors, but did not alter the affinity (\( K_d \)). These effects were dose and time dependent. Verapamil (10\(^{-6}\) M) increased the \( \beta \)-receptor density by about 13\% after 6 hours of incubation, and this increase in density reached a plateau of about 45\% after 24 hours of incubation. \( \beta \)-Receptor density increased by 15\% with 5\(\times\)10\(^{-7}\) M and by 37\% with 10\(^{-6}\) M verapamil. The increased \( \beta \)-receptors appeared to retain their normal function, as assessed by the increased spontaneous beating of the myocytes in response to applied isoproterenol. The increase in \( \beta \)-receptors was abolished by colchicine but not by cycloheximide. When the calcium ion concentration of the medium was lowered to 0.1 mM, no significant change occurred in the density of \( \beta \)-receptors compared with that in 1.8-mM Ca\(^{2+}\) medium. The results suggest that calcium antagonists increase \( \beta \)-receptors by accelerating recycling by microtubules but not by decreasing the inward calcium current. Such effects of calcium antagonists may be clinically important and promise insight into the mechanism of the withdrawal phenomenon of calcium antagonists. (Circulation 1990;81:1401–1408)

Calcium antagonists have been widely used in the clinical setting, because of their important role in the treatment of many kinds of cardiovascular disorders, such as angina pectoris, hypertension, myocardial infarction, and arrhythmia.\(^1\)\(^–\)\(^3\)

Originally, classification of calcium antagonists was based on their ability to affect the calcium inward current,\(^4\) but drugs so classified are also known to affect muscarinic and \( \alpha \)-adrenergic receptors.\(^5\)\(^–\)\(^7\) At present, the various calcium antagonists are subclassified according to their chemical structures and selectivity to various vascular smooth muscle cells.\(^8\)\(^,\)\(^9\)

The calcium channel blocking action of each drug may depend on the specific state of the calcium channel, such as resting, activated or inactivated, and the types of calcium channels that reside in various tissues.\(^5\)\(^,\)\(^10\)\(^,\)\(^11\)

Recently, the effects of calcium antagonists on channels other than those of calcium have received attention, and common pathways have been suggested in regulating \( \beta \)-receptors and calcium channels.\(^12\)\(^,\)\(^13\) This is quite interesting, because the withdrawal phenomenon of calcium antagonists along with \( \beta \)-blockade was recently reported.\(^14\) The withdrawal phenomenon of \( \beta \)-blockade has been attributed to the increased sensitivity of \( \beta \)-receptors\(^15\) to circulating catecholamines.

The withdrawal phenomenon of calcium antagonists together with \( \beta \)-blockade has led us to hypothesize that calcium antagonists may modulate \( \beta \)-receptors. The hypothesis is worth studying, because calcium antagonists are important in the treatment of cardiovascular diseases, in which \( \beta \)-receptors are also important. However, to date, investigation of the effect of calcium antagonists on cardiac \( \beta \)-receptors has been rare and little is known about the interaction between calcium antagonists and \( \beta \)-receptors.\(^16\)\(^,\)\(^17\)

We report here the effects of three calcium antagonists, verapamil, diltiazem, and nicardipine, on the \( \beta \)-receptor number and \( K_d \) of cultured cardiac myocytes and spontaneous beating. This experiment permitted us to study the direct effects of drugs on
β-receptors and enabled us to exclude possible interference attributed to changes in circulating catecholamines, which is unavoidable in an in vivo study.

**Materials**

**Cell Culture**

Cardiac myocytes were prepared from neonatal rat ventricles by the modified Bollon's method. Hearts were removed from 3–5-day-old neonatal Wistar-King rats of either sex under ether anesthesia. Ventricles of the excised hearts were minced into 1-mm³ pieces in phosphate-buffered solution (PBS). The pieces were washed three times with PBS, incubated in 0.02%-EDTA solution for 5 minutes at 37°C with shaking, and then centrifuged at 500g for 2 minutes to remove the EDTA. The pieces were then incubated in 5 ml Hanks' solution (HBSS) containing 2 mg/ml collagenase (Type IV, Cooper Biochemical, Philadelphia, Pennsylvania) for 10 minutes in a 37°C water bath with shaking at 120 rpm.

The supernatant containing erythrocytes and cell debris was discarded, and the remaining pieces were treated with 5 ml 1,000 IU/ml dispase (Godoshusei, Tokyo) in Ca²⁺- and Mg²⁺-free HBSS using a magnetic stirrer for 20 minutes. The procedure was repeated twice; the free-floating cardiac myocytes were stored in Dulbecco's modified Eagle's medium (DMEM), and this was centrifuged at 500g for 5 minutes to collect the isolated cardiac myocytes. The collected myocytes were resuspended in DMEM, seeded in plastic culture dishes, and incubated for 90 minutes at 37°C in a carbon dioxide incubator with a gas phase of 5% CO₂ in air. The fibroblasts attached to the bottom of the culture dishes after 1–2 hours, and the floating cardiac myocytes were collected by decantation.

The cell numbers were adjusted to 5×10⁵ cells/ml in DMEM supplemented with 5% fetal bovine serum (GIBCO Labs, Grand Island, New York), 10 mM HEPES, and 100 IU/ml kanamycin. One milliliter of cell suspension was inoculated into a 35-mm diameter culture dish. After 48 hours of culture, more than 70% of the cells adhered to the culture dish. Thereafter, the cultures were refed daily.

The cultured myocytes began to oscillate on the second culture day and formed monolayers by the fourth culture day. At that time, most of the myocytes beat synchronously and steadily at constant frequency (65±3 beats/min). The myocytes of the third to the fifth culture days were used for the experiments, unless otherwise specified.

**Observation of Spontaneous Beating of Cultured Myocytes**

After the equilibration of the culture environments by various maneuvers, the spontaneous beating of the myocytes was recorded through an infrared television camera by a video tape recorder with a phase-contrast microscope. The cultured myocytes were then exposed to the test drugs, and spontaneous beating was monitored 15 minutes after a drug application.

The video records were replayed later to analyze the frequency of the beating.

For the comparison between control cells and those treated with verapamil, the responsiveness to externally applied isoproterenol was assessed by the change in the spontaneous beating frequency.

Both cell groups were exposed to variable concentrations of isoproterenol, and the change in the beating frequency was normalized and plotted as a percentage of maximal response. The cells treated with 10⁻⁶ M verapamil for 24 hours were rinsed with control culture medium, and the exposure to isoproterenol was performed after almost full recovery of the beating.

**Measurements of β-Receptors**

β-Receptors were identified by radiobinding assay according to the method of Limas and Limas. Radioactive ligand, [³H]CGP-12177 (specific activity 42.0 Ci/mmol, Amersham/Searle, Zurich, Switzerland), is hydrophilic and specifically labels cell surface-bound β-receptors.

The myocytes were washed on the fourth culture day three times with assay buffer (0.25 M sucrose, 10 mM MgCl₂, and 50 mM Tris-HCl, pH 7.4) and were scraped off with a rubber policeman to make a cell suspension. The cell suspension was incubated with 1 ml medium containing [³H]CGP-12177 at concentrations of 0.25–10 nM for 16 hours at 4°C. A threefold volume of ice-cold Tris MgCl₂ buffer was then added to each dish, and the samples were filtered through GF/C filters (Whatman, Clifton, New Jersey). After washing three times with 6 ml ice-cold buffer, the filters were counted in 5 ml Aquasol-2 (New England Nuclear, Boston, Massachusetts). The measurement was repeated.

Nonspecific binding was defined by using 10⁻⁴ M propranolol. Although 10⁻⁵ M propranolol was enough for defining nonspecific binding in the ligand concentrations used in the present experiments, 10⁻⁴ M propranolol was needed when higher concentrations of ligand were used in the preliminary experiments (data not presented). Hence, 10⁻⁴ M propranolol was used throughout the experiments.

To study the time- and dose-dependent effects of verapamil on β-receptors, cells were treated on the fourth culture day with various concentrations of verapamil between 10⁻⁶ M and 5×10⁻⁹ M for 2, 6, 12, 24, and 48 hours. Thereafter, the test dishes were transferred to an ice-cold water bath. A threefold volume of cold buffer was added to each dish. The cells were washed three times and resuspended in assay buffer. Then, 10 nM [³H]CGP-12177 was added to the cell suspensions, and radioactivity was counted after 16 hours of incubation at 4°C as described above.

The effect of calcium ion concentration of the culture medium on β-receptor density was also studied with four different calcium ion concentrations, which were 0.1, 0.9, 1.8, and 3.6 mM. The action of
verapamil was also investigated in the environments of four calcium ion concentrations.

To evaluate the effects of cycloheximide and colchicine on the increase in the number of \( \beta \)-receptors induced by verapamil, the cells were pretreated on the fourth culture day with \( 10^{-5} \) M cycloheximide or \( 10^{-6} \) M colchicine for 1 hour and then exposed to \( 10^{-6} \) M verapamil for 24 hours.

For the measurements of \( \beta \)-receptors, the cells were used on the fourth culture day unless otherwise stated.

The protein content of the cells was measured by the Builett method.

For all of these measurements, control data were obtained from the sister culture in which cells were harvested in DMEM during the same period as cells treated with the test drugs.

**Drugs**

Verapamil, nicardipine, and diltiazem were kind gifts from Eizai Pharmaceutical Company (Tokyo), Yamanouchi Pharmaceutical Company (Tokyo), and Tanabe Pharmaceutical Company (Osaka, Japan), respectively.

**Statistical Analysis**

The data are expressed as mean±SD. Statistical analysis was performed with the Student's \( t \) test for unpaired samples or Wilcoxon’s ranked sum test. Results were considered significant at a \( p \) value less than 0.05.

**Results**

**Effects of Calcium Antagonists on Spontaneous Beating of Cultured Cardiac Myocytes**

The myocytes steadily beat synchronously (65±3 beats/min) on the fourth culture day. The frequency of this spontaneous beating was accelerated by isoproterenol and increased concentrations of calcium in the culture medium. The beating was suppressed dose dependently by calcium antagonists and \( \beta \)-blockade. Verapamil decreased the beating frequency by 15% at \( 5 \times 10^{-8} \) M, by 55% at \( 10^{-7} \) M, and by 100% within 5 minutes at \( 10^{-6} \) M. Nicardipine (\( 10^{-7} \) M) also suppressed the beating completely, although the corresponding concentration was one tenth that of verapamil (Figure 1). Diltiazem at \( 10^{-6} \) M only partially suppressed the beating after 24 hours of treatment.

The spontaneous beating recovered from suppression to almost the control value within 20 minutes after removal of the drugs, indicating that the effect was reversible.
TABLE 1. Effect of Calcium Antagonist on β-Receptors

<table>
<thead>
<tr>
<th>Drugs (10^{-6} M)</th>
<th>n</th>
<th>R_{max} (fmol/mg protein)</th>
<th>K_d (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>5</td>
<td>118±18</td>
<td>3.0±1.0</td>
</tr>
<tr>
<td>Verapamil</td>
<td>5</td>
<td>167±23</td>
<td>2.6±0.8</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>4</td>
<td>153±16</td>
<td>2.4±0.8</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>4</td>
<td>148±14</td>
<td>2.4±1.0</td>
</tr>
</tbody>
</table>

Results are mean±SD.

Measurement of β-Receptors

As shown in Figure 2, [3H]CGP-12177 bound to cardiac myocytes in a saturable manner, and Scatchard plots revealed a single class of binding sites with a K_d of 3.0±1.0 nM. The calculated β-receptor density was about 118±18 fmol/mg protein in the control. The three calcium antagonists (verapamil, nicardipine, and diltiazem), at 10^{-6} M, increased the number of assayable β-receptors of the cells after 24 hours of treatment, without altering the affinity to the [3H]CGP-12177. The number of β-receptors was increased from 118±18 to 167±23 by verapamil, to 148±14 by nicardipine, and to 153±16 fmol/mg protein by diltiazem at 10^{-6} M. These increases in receptor number were not significantly different among the three calcium antagonists. Effects of these calcium antagonists on cardiac β-receptors are summarized in Table 1.

The effect of verapamil, a prototype calcium antagonists, was further examined to characterize its effect on cardiac β-receptors. The time course of verapamil’s effect on β-receptor number was studied by increasing the duration of treatment from 2 to 48 hours. The β-receptor number did not change significantly at 2 hours of treatment (0.2% increase), but it increased significantly at 6 hours (13.3% higher than control) and reached a plateau (44.8% higher than control) at 24 hours. The number of β-receptors increased significantly from 118±18 fmol/mg protein to 167±23 (p<0.01) at 24 hours of treatment with 10^{-6} M verapamil with no change in the affinity (control K_d 3.0±1.0; verapamil K_d 2.6±0.8) as shown in Figure 3.

The increase induced by verapamil achieved a plateau at 24 hours, and no further augmentation was observed during prolonged treatment. Thus, in the following experiments, the cells were exposed to verapamil for 24 hours unless otherwise specified. Washing out the effect of verapamil on β-receptors was also studied. The increased β-receptor number returned to its sister culture (control) value 24 hours after removal of the verapamil (data not shown). The increase in the number of β-receptors also depended on the concentration of the applied verapamil.

As shown in Figure 4, the increase in β-receptor number was 10% at 10^{-7}, 15% at 5×10^{-7}, 37% at 10^{-6}, and 42% at 5×10^{-6} M verapamil. However, at concentrations of verapamil higher than 10^{-5} M, cultured myocytes were partly detached from the bottom of the dishes, suggesting cell damage due to intoxication.

Function of Increased β-Receptors

According to previous studies,22–24 cardiac ischemia and exposure of cardiac cells to β-blockade induce an
increase in the number of β-receptors on the cardiac cell surface, and the increased β-receptors seem to have normal function as evaluated by isoproterenol-stimulated adenylate cyclase activity.

To assess the function of the β-receptor increase by verapamil, we investigated whether the increase in β-receptor number was accompanied by an augmented physiological response. To do this, we measured the change in beating frequency in response to externally applied isoproterenol.

In the control cultures, the mean increase in the beating frequency was 11% at $5 \times 10^{-8}$, 17% at $10^{-8}$, 29% at $5 \times 10^{-8}$, 84% at $10^{-7}$, and 142% at $5 \times 10^{-7}$ M isoproterenol, respectively (Figure 5). At $10^{-6}$ M isoproterenol, the beating was irregular and arrhythmic, so those data were discarded.

In the cells treated with $10^{-6}$ M verapamil for 24 hours, the percent increase in beating frequency was augmented compared with that of the control. The increases were 26% in verapamil-treated cells and 17% in control cells at $10^{-8}$ M isoproterenol, and the increases were 142.8% in verapamil-treated cells and 84.8% in control cells at $5 \times 10^{-7}$ M isoproterenol.

Consequently, as shown in Figure 5, verapamil shifted the normalized dose-response curve to the left, suggesting that the cells became hypersensitive in response to isoproterenol.

**Mechanism of Increase of β-Receptor Density by Verapamil**

To clarify the mechanism of action of verapamil, the effect of cycloheximide, colchicine, and calcium ion concentration in the culture medium were investigated. Pretreatment of the cultured myocytes with $10^{-5}$ M cycloheximide inhibited the leucine uptake by more than 90% (not shown); however, the drug, by itself, did not significantly alter the number of β receptors. In the presence of cycloheximide, verapamil still increased the number of β-receptors, demonstrating that cycloheximide could not suppress the effect of verapamil on the receptors (Figure 6).

However, $10^{-6}$ M colchicine, which is a microtubule assembly inhibitor, significantly inhibited the increase of β-receptors by verapamil, although colchicine, by itself, resulted in little decrease in the number of β-receptors (Figure 6).

The concentration of calcium ion in the cultured medium was varied to test whether the calcium ion had any contribution on the measured β-receptor density.

The calcium ion concentration of the medium was lowered to 0.9 and 0.1 mM. In the medium containing 0.1 mM Ca$^{2+}$, cultured myocytes did not beat spontaneously as did the cells treated with verapamil. In such conditions, the measured density of β-receptors was 90±10 in 0.1, 9.8±10 in 0.9, 91±6 in 1.8, and 88±8 fmol/mg protein in 3.6 mM Ca$^{2+}$ medium, respectively. Hence, little difference was found in the measured β-receptor density among the four different calcium ion concentrations in the absence of verapamil as illustrated in Figure 7.

An augmentative effect of verapamil on β-receptors was consistently observed regardless of the concentration of calcium ion in the culture medium, which was statistically significant (Figure 7). Thus, its action did not seem to depend on the calcium concentration of the medium.
As for the density of \( \beta \)-receptors, Hedberg et al.\(^{16} \) observed a 46–65% increase in the number of \( \beta \)-receptors (\( \beta_1 \) and \( \beta_2 \)) in human atria treated with calcium antagonists. Feldman et al.\(^{17} \) reported that verapamil acted as an antagonist on \( \beta \) receptors and inhibited the specific binding of \(^{125}\)Iiodocyano-pindolol in human lymphocytes. They also found that oral treatment with verapamil in healthy subjects induced an increase in the affinity of \( \beta \)-receptors on lymphocytes for the agonist isoproterenol, although they did not observe any change in the receptor density.

The discrepancy between our findings and those of Feldman et al.\(^{17} \) regarding the \( \beta \)-receptor density may be attributed to differences in the experimental material and the system. Their material was not cardiac myocytes but human lymphocytes and were studied in healthy subjects, where a minute change in the concentration of serum catecholamine due to environmental change, such as in posture, could alter the receptor density as they described in another study.\(^{25} \)

The life cycle of \( \beta \)-receptors has not been well elucidated.\(^{24,26} \) There appear to be at least two different pathways for the process of recovery of \( \beta \)-receptors from down-regulation induced by \( \beta \)-agonists, one is the process that requires protein synthesis and the other does not.

Marsh et al.\(^{27} \) demonstrated, on chick heart cells, that the recovery of \( \beta \) receptors from down-regulation was inhibited in the presence of cycloheximide, whereas Limas and Limas\(^{20} \) reported that recovery does not require any protein synthesis in rat cardiac cells. Doss et al.\(^{28} \) observed that whether or not the recovery process needed a protein synthesis seemed to depend on the experimental situation in an astrocytoma cell line. That is, when the cells were preconfluent, cycloheximide could not inhibit the recovery process from down-regulation, but when the cells were confluent, cycloheximide did inhibit the recovery process of \( \beta \)-receptors.

Hence, no definite conclusion regarding the role of protein synthesis in the recovery process of \( \beta \)-receptors has been established until now.\(^{20,27–29} \)

In our experiments, cycloheximide, an inhibitor of protein synthesis, failed to suppress the action of verapamil, suggesting little involvement of protein synthesis in the drug action. This result is consistent with the results of Hertel and Staehelin\(^{29} \) and Limas and Limas\(^{20} \) who demonstrated the increase in \( \beta \)-receptors under conditions of no protein synthesis. However, we observed the up-regulation of \( \beta \)-receptors rather than the recovery process from down-regulation; thus, we should be cautious in directly comparing the two processes.

The effect of verapamil was suppressed by colchicine in the present experiments. Because colchicine is known to suppress the function of microtubules, verapamil may modulate the recycling process by microtubules resulting in increases in the cell-surface bound \( \beta \)-receptors without requiring protein synthe-
sis. The contribution of microtubules in regulating receptor recycling was also suggested by other investigators.20,27

One may argue that the following two possibilities should also be considered in the mechanism of action of calcium antagonists. One is the possibility of the block of tonic internalization of β-receptors similar to β-blockade.24 The other is that modulation of membrane fluidity through the interaction with membrane lipid30,31 may regulate the increment of assayable β-receptors. However, these effects of β-antagonistic action by calcium antagonists17 and interaction with the lipid bilayer were observed at higher concentrations (10⁻⁴ M) than the concentration of verapamil (10⁻⁶ M or less) used in the present experiments. Thus, the contribution of the two mechanisms to the augmentation, if any, of β-receptors could be excluded in our experiments.

The relation between the effect of calcium antagonists on the β-receptor density and that of blocking calcium current should be discussed. Hertel and Staehelin29 observed in C6-glioma cells that increased intracellular calcium ion activity inhibited the reappearance of the receptors but had no effect on their disappearance. In the present experiments, lowering the concentration of calcium ions in the medium to 0.1 mM induced no significant change in the β-receptor density.

The level of the intracellular calcium ion concentration attained by lowering the concentration of the calcium ion in the medium to 0.1 mM may not be comparable to that induced by calcium antagonists in 1.8 mM Ca²⁺ medium. However, it may not be far from that, because the myocytes ceased to beat spontaneously as did the verapamil-treated cells. Furthermore, no change was observed in the density of β-receptors in the four different calcium ion concentrations of the culture medium: 0.1, 0.9, 1.8, and 3.6 mM. The augmentation of receptor density by verapamil was still clearly seen in these conditions. Hence, we conclude that verapamil’s ability to increase β-receptor density in our study does not seem solely attributable to a decrease in the intracellular calcium ion concentration, which was induced by blocking the calcium current, but due to some other action or actions. We admit, however, that we do not know the exact level of the intracellular calcium ion concentration in the presence of calcium antagonists, because we did not measure it directly. Further detailed analysis is necessary to explore the role of intracellular calcium ion concentration in the recycling of β-receptors in cardiac cells.

**Clinical Implications**

The clinical implications of the present study are important, because calcium antagonists are widely used in daily clinical treatment. There have been a few reports concerning the withdrawal phenomenon of calcium antagonists, namely, the occurrence of severe hypertension, angina pectoris, and acute myocardial infarction.14,32 Although the mechanism of the phenomenon has not been fully detailed,23 the increase in the number of β-receptors could contribute to the occurrence of the phenomenon in patients with cardiovascular disorders. In the case of β-blockade withdrawal phenomenon, the increase in β-receptor density is considered to be a possible mechanism.15 Likewise, sudden termination of treatment with calcium antagonists may elicit an exaggerated reaction to the serum catecholamine resulting in the induction of severe hypertension and angina pectoris, particularly on occasions in which long-term combination therapy of both calcium antagonists and β-blockade was discontinued. Thus, the termination of either calcium antagonists or β-blockade should be done cautiously in the daily clinical setting.

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