Mechanisms of β-Adrenergic Receptor Regulation in Cultured Chick Heart Cells

Role of Cytoskeleton Function and Protein Synthesis

James D. Marsh, Daniel Lachance, and Donghee Kim
From the Cardiovascular Division, Department of Medicine, Brigham & Women's Hospital and Harvard Medical School, Boston, Massachusetts

SUMMARY. To examine mechanisms by which cardiac tissue regulates the β-adrenergic receptor and physiological response to β-adrenergic agonists, we studied the effects of cytoskeletal disrupting agents and inhibition of protein synthesis on receptor properties and contractile response to isoproterenol in intact cultured ventricular cells from embryonic chick heart. Thirty minutes of exposure of intact cells to 1 μM isoproterenol produced loss of the high-affinity state (Kd = 4.5 ± 1.5 nM) of the receptor found in cell membranes with no loss of total receptor number, whereas there was concomitant decline in the contractile response to 1 μM isoproterenol to 41 ± 16% (so) of control. Contractile response recovered within 60 minutes of agonist removal to 78 ± 11% of initial response. There was concomitant recovery of the high-affinity state of the receptor, so that 1 hour after agonist removal there was 72% of the initial proportion of high-affinity receptors. This desensitization of the contractile response, as well as recovery after agonist removal, was markedly blunted by preincubation with cytochalasin B so that contractile responsiveness to isoproterenol was maintained at 77 ± 13% of the initial response. Colchicine (10 μM) was without effect on the first 30 minutes of agonist-induced desensitization. More prolonged agonist exposure (1 μM isoproterenol for 24 hours) produced colchicine-sensitive loss of receptors from intact cells to 40% of control levels. Full recovery of receptor number occurred over 72 hours; this was completely blocked by cycloheximide (P < 0.01). Thus, rapid desensitization and resensitization of the β-receptor-mediated contractile response is associated with alterations in high-affinity agonist binding and appears to be modulated by microfilaments. Receptor down-regulation is dependent on functional microtubules, and recovery of these receptors after agonist removal requires protein synthesis. (Circ Res 57:171-181, 1985)

TUMOR cell lines and erythrocyte membranes have been studied with respect to the β-receptor-guanine nucleotide regulatory subunit-adenylate cyclase system, and have been utilized to examine phenomena involved in the transduction of a hormone signal to physiological response (Watanabe et al., 1982; Lefkowitz et al., 1983). The mechanism by which the cardiac β-adrenergic receptor is regulated has been studied in much less detail, although it is likely to be an important process in the pathophysiology of several disease states including congestive heart failure (Bristow, et al., 1982) and acute myocardial ischemia (Mukherjee et al., 1982; Krause et al., 1982, Barber et al., 1983) in which there is altered local concentrations of catecholamines. This exposure to high concentrations of agonist can alter β-adrenergic receptor properties in cardiac tissue (Marsh et al., 1980; Bobick et al., 1981). Recently, Limas and Limas (1984) reported regulation of antagonist binding to the β-receptor in freshly dissociated heart cells by catecholamine exposure, and demonstrated a role for the cytoskeleton in agonist-induced alterations in receptor expression on the cell surface. Previous investigations from this laboratory have demonstrated rapid desensitization to the positive inotropic effect of isoproterenol on ventricular tissue without alteration in the properties of the β-adrenergic receptor as determined by antagonist binding. However, there was rapid uncoupling of the β-receptor from adenylate cyclase that developed concomitantly with contractile desensitization (Marsh et al., 1980). Furthermore, cultured heart cells derived from embryonic chick ventricle show rapid, physiological, contractile desensitization to the effects of isoproterenol that are dependent on concentration of agonist and duration of exposure (Marsh et al., 1982).

An important gap exists in the understanding of the mechanism by which β-adrenergic receptor occupancy and subsequent events modulate the physiological response to catecholamines in cardiac tissue, despite important advances in this understanding in other model systems (Hardin, 1983). Thus, in several cultured tumor cell systems, two stages of desensitization to catecholamines have been reported (Walco et al., 1983; Toews and Perkins, 1984). This has previously not been fully elucidated in cardiac tissue (Harden, 1983), although Limas...
and Limas (1984) have reported an early desensitization step. If, indeed, the cardiac β-receptor is modulated in a process involving multiple stages, little is known of the physiological function of the regulated receptors.

Hypotheses regarding mechanisms of cardiac β-receptor modulation can be formulated based on recent advances in the study of β-receptor modulation in several model systems including tumor cell lines and erythrocyte membrane preparations (for reviews see Harden, 1983; Lefkowitz et al., 1983). However, extrapolating mechanisms of receptor regulation from relatively simple model systems of erythrocytes or tumor cell lines to mechanisms of β-receptor regulation in cardiac tissue is hazardous, since there appear to be major differences in mechanisms that regulate the β-receptor in systems as seemingly similar as frog and turkey erythrocytes (Stadel et al., 1983a, 1983b).

Furthermore, an important limitation of previous work in model systems and even in cardiac tissue has been that no physiological response other than adenylate cyclase activation or cyclic adenosine monophosphate (cAMP) accumulation has been measured. It has been implicitly assumed that there is a direct and unvarying relation between cAMP production and cell function, including contractility, for the case of cardiac tissue. Particularly for studies on the recovery of the desensitized β-receptor, the functional status of the "recovered" receptor has been unknown, but a priori cannot be assumed to be normal. We now combine observations on physiological contractile performance of cardiac cells during agonist-induced desensitization and recovery from desensitization with concurrent studies on receptor properties of the same cardiac cell preparation under identical conditions.

**Methods**

**Tissue Culture**

Monolayer cultures of beating chick embryo ventricular cells were prepared as previously described (Marsh et al., 1982). Briefly, 10-day-old chick embryo hearts were removed under sterile conditions, and the ventricle was cut into 0.5-mm fragments and placed in Ca++- and Mg++-free Hanks' solution. The ventricular fragments were gently agitated in 10 ml of 0.025% (wt/vol) trypsin (Grand Island Biological Company), and Ca++- and Mg++-free Hanks' solution at 37°C for four or five cycles of 7 minutes each. The supernatant suspensions containing dissociated cells were prepared as previously described (Marsh et al., 1983).

The suspension of cells was diluted to 5 × 10⁶ cells/ml and placed in plastic tissue culture dishes containing 25-mm circular glass cover slips (VWR; thickness #2). The cells prepared for ligand-binding studies were placed directly into the tissue culture dishes. Cultures were incubated in a humidified 5% CO₂-95% air atmosphere at 37°C. Confluent monolayers in which at least 70% of the cells were contracting developed by 2 days in culture. All studies on contractility were done 3 or 4 days in culture, as well all ligand-binding studies; control experiments showed no difference in results between day 3 and day 4. For cultures to be used for contractility studies, plastic microspheres 2-3 μm in diameter (3M Company) were added to the cultures on the 2nd day of growth. The plastic microspheres became attached to the cell surface and were moved by contraction of individual cells in the monolayer. This provided an improved image for contractile recording and measurement.

For experiments on long-term receptor recovery, cells were changed to serum-free culture medium after exposure to isoproterenol and washing. The serum-free medium was identical to serum-containing culture medium except that the 6% FCS was omitted and triiodothyronine (1 × 10⁻¹⁰ M), selenium (1 × 10⁻⁷ M), insulin (1 × 10⁻⁸ M) and transferrin (5 µg/ml) were added (Libby, 1984). All of these agents are normal constituents of serum. Concentrations were chosen that maintained levels of protein synthesis near levels achieved with FCS supplement and maintained long-term contractile activity and morphological integrity (Libby, 1984).

Ligand-binding experiments and contractility measurements were conducted in HEPES (4 mM)-buffered physiological salt solution containing (mM): Na⁺, 144; K⁺, 4.0; Ca²⁺, 0.97; HCO₃⁻, 18; Mg²⁺, 0.8; and Cl⁻, 131. The solution for contractility studies also contained 1% FCS to improve the regularity of beating. Qualitatively similar results were obtained in the absence of any FCS.

**Measurement of Contractility**

A glass cover slip with attached heart cell monolayer was placed in a specially designed perfusion chamber that permitted a continuous flow of a perfusing medium over the monolayer. The chamber was placed on the stage of an inverted phase contrast microscope (Leitz Diavert) enclosed in a Lucite box with controlled temperature (37 ± 0.5°C). The inlet to the perfusion chamber was connected by tubing to four syringe pumps so that the culture medium could be sequentially perfused with a variety of pre-warmed test media. Flow characteristics of the chamber were estimated by indocyanine green dye washout. Using a flow rate of 0.96 ml/min, medium bathing a cell on the center of a coverslip was exchanged with a time constant of about 15 seconds. Media samples from the chamber were analyzed (Instrumentation Laboratories pH-gas analyzer) and consistently found to have a pH greater than 100 and pH = 7.3. The optical apparatus was supported by an air table to damp building vibrations, and the cells were magnified using a 40x objective. The image was monitored by a low-light level silicon TV camera (Dage 650 SXX) attached to the microscope observation tube with a 2x coupler. The TV camera video output was connected to a video motion detector (Colorado Video 633) and displayed on a Conrac TV monitor. The total magnification of the image on the monitor screen was 2000x. The TV camera had an interface defeat so that the image was composed of 262 raster lines. The motion detector monitored a selected raster line segment and pro-
vided new position data every 16 msec for an image border of a microsphere within the monolayer moving along the raster line. The analog voltage output from the motion detector was filtered at 15 Hz with a 48 dB/octave low-pass active filter and was calibrated to indicate actual microns of motion. The analog tracing was recorded with a physiological recorder. Rate and amplitude of contraction were recorded on the strip chart periodically. Previous studies from our laboratory (Barry et al., 1975; Biedert et al., 1979; Marsh et al., 1980) and from another laboratory (Clusin, 1981) have demonstrated that changes in amplitude and velocity of wall motion may be used to quantify the effects of a variety of interventions on cell contractility.

**Protocol for Contractility Measurement**

To determine the contractile response of cultured cells to isoproterenol, a coverslip with a spontaneously contracting monolayer was continuously superfused with 0.6 mM Ca++ medium for 10 minutes, and the amplitude of contraction of a cell was continuously recorded. When the response had stabilized, the monolayer was exposed to various test solutions. Response to various concentrations of isoproterenol or calcium reached a plateau by 3–5 minutes. For each cell studied, the inotropic response to isoproterenol was expressed as the percentage of the response to 3.6 mM Ca++, a Ca++ concentration that elicited a maximal inotropic response, so the results from individual experiments could be compared. Loading conditions for contraction caused by attachment of the cell to the coverslip and other cells, as well as the viscoelastic properties of each cell, may vary from cell to cell. However, by expressing the contractile response of the cell as a percentage of its maximal response (Marsh et al., 1982), these differences in loading conditions are taken into account, and each cell can serve as its own control. Previous studies from this laboratory have demonstrated that this method of assessing the inotropic state of cells in a monolayer correlates very closely with other physiological parameters expected to relate to the inotropic state, such as alterations in monovalent and divalent cation fluxes (Biedert et al., 1979) and response to altered oxygen tension (Barry et al., 1980). The contractility measurements were made on only one cell per coverslip. Several coverslips were used from one plating; all experimental points comprise results from two or more platings.

**Iodopindolol**

The pure L-isomer of pindolol was radioiodonated and purified by a modification of the method of Barovsky and Brooker (1980) as previously described (Marsh and Smith, 1984).

**Cell Suspensions**

To minimize nonspecific binding of iodopindolol to culture plates and interstitial space, binding experiments were conducted in suspensions of intact cells. Culture medium was aspirated from tissue culture plates and the cultures were washed twice with 4 ml of ice-cold assay buffer. Sheets of cells were carefully scraped from the culture dishes with a rubber scraper and collected in a small volume of assay buffer. The intact cells were centrifuged at 500 g at 4°C, resuspended at a final concentration of approximately 0.3 mg protein/ml, and used immediately in the assay.

When membranes of cells were to be prepared, plates were washed with assay buffer; then the plates were vigorously scraped with a rubber scraper and cellular material was collected in a small volume of buffer. Cells were further disrupted by a Polytron homogenizer (5 sec, 50% speed). Final homogenization and resuspension was accomplished by 10 strokes of a tight-fitting Dounce homogenizer. Cells used in radioligand binding studies were grown in the presence of 25Se-methionine to permit normalization for protein content of each sample using double-label counting techniques. Protein concentrations were estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard. β-Adrenergic receptor properties were assessed by adding 900 μl of intact cell suspension or cell membrane to a mixture containing the desired concentration of iodopindolol (IPIN) and, depending on the experimental design, β-adrenergic agonist or antagonist. To prevent oxidation, catecholamines were prepared and maintained in 1 mM ascorbate. Control experiments showed that ascorbate did not perturb IPIN binding. The binding reaction was conducted for varying periods of time at 37°C. Binding was terminated by adding 3 ml of wash buffer (potassium phosphate, pH 7.4) to the assay tube, followed by rapid filtering under controlled vacuum through Whatman GF/C filters. The filters were washed twice with an additional 5 ml of buffer. Approximately 8 seconds were required to terminate the binding reactions and wash the filters. The filters were dried under high vacuum and counted at 69% efficiency in an LKB γ-counter. Simultaneously determined 125I counts (for IPIN binding) and 32P counts (to correct for inter-tube variation in cell density) were analyzed on a DEC PDP 11/23 computer.

During the course of these investigations, the highly hydrophilic β-receptor antagonist ligand CGP28392 became available. Because of its lipophobicity, it can be utilized to identify β-receptors preferentially on the surface of intact cells (Staehelin and Hertel, 1983). Moreover, it permitted the use of binding assays on cells attached to culture plates by the same procedure used for IPIN binding to cell suspensions. Binding was terminated by rapid washing of plates with 5 ml × 3 of wash buffer followed by cell solubilization with 1% sodium dodecyl sulfate and 10 mM sodium borate. Radioactivity in aliquots of the dissolved cells was assessed by a liquid scintillation counter (Packard 3330).

CGP12177 bound to intact cells with high affinity (Kp 0.16 ± 0.06 nM n = 6) and saturaBly. Computer modeling of equilibrium binding isotherms for CGP12177 as well as isoproterenol displacement curves revealed a single class of binding sites for CGP12177 and for isoproterenol. Isoproterenol (0.1 mM) displaces all but <5% of a concentration of CGP12177 near the Kp. When full saturation-binding isotherms for IPIN and for CGP12177 were determined simultaneously under identical conditions on two aliquots of intact, suspended cells from the same culture, CGP12177 identified 99% as many antagonist-binding sites as were identified by IPIN. Four hours of exposure to 1 μM isoproterenol produced no significant change in dissociation constant for either of the antagonist ligands.

**Data Analysis**

Equilibrium-binding data and agonist displacement data were analyzed using the iterative, nonlinear, least-squares method of Munson and Rodbard (1980) on an IBM 4341 computer to solve the generalized problem of the interaction of hormone with receptor using the law of mass action. When the model is applied to the case of
separate affinity states, one assumes that these states, although interconvertible, can nevertheless be segregated. Considerable evidence supports these assumptions in \( \beta \)-adrenergic systems (Lefkowitz et al., 1983; Harden et al., 1983). Criteria used for goodness-of-fit were those of Kent et al. (1980). Each experiment was analyzed individually, and binding parameters for all replicate experiments were averaged. Simultaneous analysis of all replicate experiments was also performed; binding parameters estimated in this way were in good agreement with the values obtained by averaging.

Contractility responses and data on total receptor numbers were compared by two-sample t-test or Mann-Whitney U-test, where appropriate. Data are expressed as mean ± 1 sd.

Materials

L-pindolol was the generous gift of Dr. Joel Linden and of Sandoz Pharmaceuticals. \( ^{125} \)I was from New England Nuclear. \( ^{35} \)Se-methionine was from Amersham. Tissue culture media were from sources previously described (Marsh et al., 1982). All other chemicals were from Sigma.

Results

Contractile Response to Isoproterenol

To determine the contractile response of intact cells in monolayers to isoproterenol, cells were first exposed to 0.6 mm Ca\(^{++}\) for 5–10 minutes until a stable baseline of contractile amplitude was observed. Then, so that their maximal inotropic responsiveness could be determined (Marsh et al., 1982), cells were superfused with physiological salt solution containing 3.6 mm Ca\(^{++}\). Contractility was then permitted to return to baseline in 0.6 mm Ca\(^{++}\). Subsequently, the cells were exposed to 1 \( \mu \)M isoproterenol, a concentration on the plateau of the concentration-effect curve (Marsh et al., 1982). Amplitude of contraction increased and reached a plateau within 3–5 minutes. The response was typically about 85% as great as that observed with exposure to 3.6 mm Ca\(^{++}\). There was no alteration in beating rate (Marsh et al., 1982) for these slow channel-dependent cells. As the cells were continuously superfused with 1 \( \mu \)M isoproterenol, contractile response declined and reached a new plateau by 30 minutes of exposure. The response to isoproterenol at 30 minutes was 43 ± 16% of the peak amplitude of contraction observed at 3–5 minutes (n = 14). Thus, the spontaneously contracting cells showed rapid desensitization in their contractile response to isoproterenol. We have previously reported (Marsh et al., 1982) that this desensitization is specific for the contractile response to isoproterenol and does not reflect nonspecific deterioration of the cell preparation. Cells inotropically desensitized to isoproterenol maintained full inotropic responsiveness to a noncatecholamine-positive inotropic agent such as calcium.

Alterations in Receptor-Binding Properties

There are several mechanisms by which cells may attenuate their specific inotropic response to \( \beta \)-adrenergic agonists, including alterations in agonist-binding properties of the receptor, alterations in agonist receptor expression in the sarcolemma, alterations in phosphodiesterase activity, or alterations in protein kinase activity. To test the hypothesis that the rapid physiological desensitization is associated with alterations in agonist binding properties, we examined agonist- and antagonist-binding properties of cultured cell membranes in control cells and in cells that had been exposed to isoproterenol for 30 minutes and then washed free of agonist. Previous work from this laboratory has demonstrated that, in membranes of the cultured chick embryo ventricular cells and also, in intact cells, two affinity states for agonist can be identified (Marsh and Smith, 1984). Furthermore, we demonstrated that occupancy of the high-affinity state of the \( \beta \)-receptor by agonist correlated very closely with the increase in contractile state and cAMP production, supporting the hypothesis that occupancy of the receptor state capable of coupling to the guanine nucleotide regulatory subunit (\( \gamma \)) and activating adenylate cyclase was the event that initiated the contractile response. Cell membranes were prepared, washed three times to remove endogenous guanine nucleotides, and the ability of various concentrations of isoproterenol to displace 50 pm IPIN was determined. Assays were conducted at 37°C for 5 minutes. Computer analysis (Monson and Rodbard, 1980) of displacement curves permits estimation of agonist and antagonist affinity and revealed a single affinity state for antagonist binding with \( K_D = 39 \pm 12 \) pm. A two-site model for agonist binding was preferred over a one-site model (n = 4, P < 0.05). As summarized in Table 1, the dissociation constant for high-affinity agonist binding was 4.5 nm, whereas that for low-affinity agonist binding was 0.36 \( \mu \)M. Forty-two ± 19% of the receptors were in the high-affinity state. To the extent that isoproterenol may not be in equilibrium with the low-affinity site, the apparent \( K_D \) overestimates the true \( K_D \).

Intact cells were desensitized by incubation with 1 \( \mu \)M isoproterenol for 30 minutes at 37°C followed by washing two times and preparation of membranes. Analysis of 18-point agonist displacement curves revealed that, compared to control cells, the curve was steeper, and in each case the number of receptors estimated to be in the high-affinity state was zero (n = 3). There was no alteration in the total number of antagonist-identifiable receptors.

### Table 1

| Dissociation Constants for Binding to Cell Membranes |
|-----------------|-----------------|-----------------|-----------------|
|                | K<sub>1</sub> (μM) | K<sub>2</sub> (μM) | K<sub>3</sub> (μM) | R<sub>2</sub>/R<sub>1</sub> |
| Control         | 39 ± 12          | 4.5 ± 1.5       | 0.36 ± 0.096     | 0.42 ± 0.19     |
| 30 Min agonist exposure | 39 ± 12          | 0.14 ± 0.03     | 0.42 ± 0.19      |

* Dissociation constants for iodopindolol and for isoproterenol binding to high-affinity state and low-affinity state, respectively (mean ± sd).

† Fraction of receptors in high-affinity state.
FIGURE 1. Isoproterenol competition curves for receptors in cell membranes. IPIN binding was determined in the presence of indicated concentrations of isoproterenol in this typical experiment. Intact cells were exposed to isoproterenol for 30 minutes, washed, and membranes prepared (desensitized, ○). Recovered cell (•) were exposed to isoproterenol for 30 minutes, washed, and incubated 1 hour in the absence of isoproterenol, and membranes were prepared. For comparison, displacement from membranes of cells not exposed to isoproterenol is shown (□). The dissociation constant for isoproterenol for these experiments are as follows: controls cells $K_d = 5.2 \text{ nM}$, $K_d = 429 \text{ nM}$, $\% R_m = 50$; desensitized cells $K_d = 170 \text{ nM}$, $R_m = 0$; recovered cells $K_d = 7.0 \text{ nM}$, $K_d = 279 \text{ nM}$, $\% R_m = 38$.

compared to control cells (Fig. 1). The same results were found if cells were desensitized by exposure to isoproterenol for 1 hour ($n = 4$). Thus, concomitant with decline in inotropic response to a $\beta$-adrenergic agonist there is loss of agonist binding to the high-affinity state of the $\beta$-receptor in membranes.

Receptor Down-Regulation

To test the hypothesis that prolonged exposure of intact cells to a $\beta$-adrenergic agonist would produce loss of total antagonist-identifiable receptors, intact cells were exposed to graded concentrations of isoproterenol ($10^{-10}$ M to $10^{-6}$ M) for 1-24 hours. The cells were then washed with physiological salt solution, and the number of binding sites identifiable by IPIN in intact cells was determined. Control cells were treated in identical fashion in each case, except that they were exposed to buffer with ascorbate only. $^{125}$I-pindolol binds to the $\beta$-receptor on intact cells in a saturable and stereospecific fashion at $37^\circ$C (Marsh and Smith, 1984). Control saturation curves showed that in cells exposed to $1 \mu$M isoproterenol for 24 hours, there was no alteration in $K_d$ for antagonist ($P > 0.05$). Therefore, a single concentration of IPIN was used to estimate the total number of binding sites on the cell surface for both control and desensitized cells. The concentration chosen was 200-250 pm, which is at least 5 times the $K_d$, an essentially saturating concentration. Figure 2 demonstrates that exposure of intact cells to agonist produced a time- and concentration-dependent loss of $\beta$-adrenergic receptor sites identified by IPIN on intact cells. Thus at 1 hour, $10^{-9}$ M isoproterenol caused no discernible receptor loss, but after 24 hours of exposure to $10^{-6}$ M isoproterenol, the total number of receptors declined to 40% of control. Over this time course there was no charge in $\beta$-receptor number in control cells.

Modulation of Receptor Down-Regulation

Microtubules have been reported to play a role in receptor regulation in a variety of tissues. To determine whether cardiac $\beta$-receptor down-regulation is altered by this component of the cytoskeleton, we preincubated intact cells with $1 \mu$M colchicine, then exposed them to $1 \mu$M isoproterenol plus colchicine for 4 hours, and analyzed the number of $\beta$-receptors identifiable in the intact cell, as described above, using IPIN as ligand ($n = 3$ experiments). Control cells were exposed to colchicine alone or isoproterenol alone. Colchicine ($1 \mu$M) exposure produced no significant change in the number of $\beta$-receptors identified (5.05 ± 1.63 fmol/mg for control cells; 4.79 ± 0.79 fmol/mg for colchicine-exposed cells, $P > 0.05$). Four-hour exposure to $1 \mu$M isoproterenol caused a loss of 3.72 fmol/mg receptors, compared with control ($P < 0.05$). If cells were preincubated in $1 \mu$M colchicine before exposure to isoproterenol, the receptor loss was 2.23 fmol/mg receptors, compared with control ($P < 0.05$ compared with isoproterenol alone). Preincubation of $10 \mu$M colchicine, followed by exposure to isoproterenol was associated with 1.01 fmol/mg receptor loss ($P > 0.05$). Thus, receptor down-regulation is inhibited in a concentration-dependent fashion by an inhibitor of microtubule function. To determine whether microfilaments might also modulate receptor down-regulation, the microfilament-disrupting agent cyto-
cholera B (10 μg/ml) was studied by an identical protocol. It had no effect on agonist-induced receptor loss.

**Recovery of Contractile Responsiveness**

Previous investigations have shown that if cells are exposed to 1 μM isoproterenol for 60 minutes, there is no recovery in the cells’ contractile response to isoproterenol during the initial 120 minutes following isoproterenol washout (Marsh et al., 1982). To determine whether there is a time point at which cells might recover their contractile responsiveness after the receptors have been converted to the low-affinity state, we exposed cells to 1 μM isoproterenol for 30 minutes, monitored their contractile response, and continued our observations for 30 minutes after isoproterenol washout. After 30 minutes of isoproterenol exposure, the contractile response to isoproterenol was 43 ± 16% of the initial response. After the cell had recovered for 30 minutes, there was substantial recovery in the inotropic responsiveness to 78% ± 11% of the initial response. Therefore, there is rapid recovery of contractile responsiveness after a relatively brief isoproterenol exposure, but not after exposure of 1 hour or more.

**Recovery of High-Affinity Binding**

To determine whether agonist binding to the high-affinity state of the β-receptor in previously desensitized cells returns with a time course similar to that for return of contractile responsiveness, cells were exposed to isoproterenol for 30 minutes, washed three times, and incubated at 37°C for 60 minutes in the absence of isoproterenol. Cell membranes then were prepared. Figure 1 demonstrates that the displacement curve in membranes of cells that have been allowed to recover from isoproterenol exposure is more shallow than that for desensitized cells. Analysis of displacement curves for membranes from recovered cells reveals that, in each case, binding of agonist is best described by two binding sites rather than one (P < 0.03), with antagonist- and agonist-binding affinities indistinguishable from those of control cell membranes (P > 0.05, n = 3). In membranes from cells desensitized for 30 minutes and permitted to recover for 1 hour, on average 31% of receptors were found to be in the high-affinity state. There is temporal similarity between decline in contractile responsiveness and loss of high-affinity agonist binding during desensitization, and recovery of high-affinity agonist binding with return of the contractile response to isoproterenol. In cells that were exposed to 1 μM isoproterenol and recovered for 30 minutes after isoproterenol removal, contractile response was ± 11% of initial response, and for cells that recovered for 60 minutes after isoproterenol removal, the proportion of high-affinity receptors in membranes had returned to 72% of the initial value. If cells were exposed to isoproterenol for 60 minutes and permitted to recover for 60 minutes, a procedure that resulted in poor recovery of contractile responsiveness (Marsh et al., 1982), then binding experiments showed equivocal results for recovery of the high affinity state in membranes: the two-state model was marginally preferred over the one-state model with P = 0.056. Thus, not only does the high-affinity state of the receptor in membranes recover after brief agonist exposure and washout, but the recovered high-affinity state of the receptor is associated with contractile response and function in a nearly normal physiological fashion.

**Modulation of Recovery from Contractile Desensitization**

To determine whether elements of the cytoskeleton modulate the contractile response to catecholamines, contractile desensitization and recovery were examined after 1 hour of preincubation with 1 μM colchicine (Table 2). Colchicine had no effect on the rapid phase of desensitization. Thus, contractile desensitization over 30 minutes in the presence of colchicine was indistinguishable from that in

### Table 2

<table>
<thead>
<tr>
<th>Condition</th>
<th>Drug present</th>
<th>ISO response*</th>
<th>ISO response*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Isoproterenol</td>
<td>41 ± 16</td>
<td>78 ± 11†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 9)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>B</td>
<td>Isoproterenol + colchicine, 10 μM</td>
<td>38 ± 14</td>
<td>85 ± 18†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 4)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>C</td>
<td>Isoproterenol + cytochalasin B, 10 μg/ml</td>
<td>77 ± 13†</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Isoproterenol</td>
<td>42 ± 8§</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochalasin B, 10 μg/ml</td>
<td></td>
<td>(n = 4)</td>
</tr>
</tbody>
</table>

* Contractile response to 1 μM isoproterenol (ISO) expressed as percent of the amplitude of response to isoproterenol 3 minutes after initial exposure (mean ± st).

† P < 0.01 compared to 30 minutes.

‡ P < 0.001 compared to condition A, 30 minutes.

§ P < 0.001 compared to condition A, 60 minutes.
control cells, as was recovery from desensitization over 30 minutes.

To determine whether microfilaments might modulate contractile desensitization or recovery, the inhibitor of actin polymerization, cytochalasin B, was examined (Table 2). For cells that were first preincubated in 10 µg/ml cytochalasin B for 1 hour, then exposed to isoproterenol, the initial inotropic response to isoproterenol was indistinguishable from that of control cells. However, the contractile desensitization seen in control cells was markedly blunted by cytochalasin B: instead of contractile response to agonist declining to 41% of control, in the absence of cytochalasin B, cells maintained 77% of their initial responsiveness. Furthermore, when cells were desensitized in the presence of isoproterenol alone for 30 minutes, and then isoproterenol was removed and the cells permitted to recover in the presence of cytochalasin B, contractile recovery was markedly reduced as well. Thus, both contractile desensitization and recovery from desensitization appear to be dependent on normal function of microfilaments. Cytochalasin D, an agent highly specific for inhibition of microfilament function, was also studied. At 1 µg/ml cytochalasin D, contractile desensitization was also blunted, with contractility remaining at 77% of control (two experiments), in concert with findings for cytochalasin B.

**Recovery from Down-Regulation**

In some β-receptor model systems, such as the frog erythrocyte, the receptor appears to be internalized during down-regulation and can be reinserted into the plasma membrane without requiring protein synthesis, whereas, in other systems, receptor shuttling does not appear to be the predominant mechanisms of receptor regulation because recovery of the β-receptor number requires protein synthesis. To determine whether cardiac tissue requires protein synthesis for recovery from β-receptor down-regulation, cells were exposed to 1 µM isoproterenol for 4 hours, washed, and permitted to recover for 72 hours in serum-free medium in the presence or absence of 1 x 10^{-4} M cycloheximide. Serum-free medium was utilized so that fibroblast proliferation would be minimal during the additional 3 days in culture. At 72 hours, intact cells were harvested, and the total number of antagonist-identifiable receptors on the cell surface was assayed with a concentration of iodopindolol at least 5 times the K_d.

Table 3 details the agonist-induced desensitization and recovery of IPIN-identifiable β-receptors in intact cells. All experiments were conducted in identical serum-free media so that the effect of growth factors did not vary. The addition of an inhibitor of protein synthesis in the absence of agonist produced a small (statistically insignificant) decline in IPIN binding from 9.4 to 8.8 fmol/mg. When cells were exposed for 4 hours to 1 µM isoproterenol, then were washed and the medium replaced, there was no recovery after 24 hours, whereas, in 72 hours, IPIN binding recovered fully (9.38 fmol/mg) if cycloheximide was absent. If receptor recovery after agonist exposure was permitted to proceed in the presence of cycloheximide, the number of receptors identified by IPIN remained markedly depressed at 3.1 fmol/mg (P < 0.05, 33% of control).

To determine whether the relatively lipophilic ligand IPIN was identifying a different pool of receptors than the very hydrophilic ligand CGP12177, the cycloheximide experiments were repeated using CGP12177 as the ligand. When β-receptors presumably on the cell surface were enumerated in serum-free medium by CGP12177, the results were qualitatively similar to those with IPIN as the ligand. Four hours of exposure to isoproterenol and 72 hours of recovery in cycloheximide results in 58% of the control number of receptors identifiable on the surface of intact cells.

**Cell Kinetics**

Recovery of down-regulated β-receptor could be due either to re-expression of receptors on the same cells or to cell division with expression of receptors only on new, naive cells. To distinguish between these possibilities, we conducted a series of experiments to examine cell number, percent myocytes, and growth characteristics after 48 and 72 hours of growth in serum and after 72 hours of growth in serum-free medium.

Cells were plated at 5 x 10^5 cells/ml (1 x 10^6 cells/well) in multiwell plates. After varying periods, cells were detached by exposure to 0.1% trypsin at 37°C for 10 minutes to produce a single cell suspension. Cells were counted and cell protein was determined (Table 4). As an additional approach to studying the rate of protein and DNA synthesis in the cultures, [3H]phenylalanine and [3H]thymidine incorporation into trichloroacetic acid-perceptible protein was studied in parallel experiments (Table 4). Observation of the cultures under phase-contrast microscopy showed that about 40–50% of initially

---

**Table 3**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Isoproterenol (4 hr)</th>
<th>Cycloheximide (72 hr)</th>
<th>Receptor* no. (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>-</td>
<td>-</td>
<td>9.4 ± 2.3</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>-</td>
<td>+</td>
<td>8.8 ± 2.5</td>
</tr>
<tr>
<td>Down-regulated/re-</td>
<td>+</td>
<td>-</td>
<td>9.4 ± 3.7</td>
</tr>
<tr>
<td>covered</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Down-regulated/re-</td>
<td>+</td>
<td>+</td>
<td>3.1 ± 1.3</td>
</tr>
<tr>
<td>recovery blocked</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Number of β-receptors identified in intact cells identified by IPIN binding 72 hours after addition of either isoproterenol or buffer, then cycloheximide or buffer, as indicated. For comparison, after cells are exposed to 1 µM isoproterenol for 4 hours (down-regulated only), the typical receptor number is 5.5 fmol/mg (see Fig 2)
plated cells appeared viable and attached to substrate. By 48-hour growth in medium containing serum, cells had undergone an average of one division. After 72 hours of subsequent growth in serum-free medium, protein and DNA synthesis had slowed and about 40% of cells had again divided. There was little change in average cell mass. If cells are grown in serum-free medium for 72 hours in the presence of 1 X 10^-4 M cycloheximide, protein and DNA synthesis rates as estimated by [3H]phenylalanine and [3H]thymidine incorporation rates were markedly depressed and there was some cell death.

To determine the ratio of myocytes to fibroblasts in the cultures at progressive stages of growth, cells grown on coverslips were fixed in Bouin's solution and stained by the Masson trichrome method. Percent myocytes was determined by the criteria of Clark (1976). After 48 hours of growth in serum-containing media, there was 93 ± 4% myocytes present. After 72-96 hours in the same medium, there was 87 ± 7% myocytes. If cultures were changed to serum-free medium at 48 hours and permitted to grow for 72 hours, as in the down-regulation/recovery experiments, 87 ± 6% of the cells were myocytes. Taken together, these studies of cell kinetics indicate that recovery of β-receptors after down-regulation is due predominantly to re-expression of β-receptors on myocytes that had been exposed to agonist.

### Discussion

The experimental observations reported here are consistent with the presence of two stages of β-adrenergic receptor regulation in spontaneously contracting cardiac cells. Initially agonist binds to the receptor, forming a high-affinity complex, Rf, that is coupled to the stimulatory subunit of guanine nucleotide regulatory protein, Ns. Adenylate cyclase is activated and a positive inotropic response results (Marsh and Smith, 1984). After agonist binding, the pool of receptors is rapidly (30-60 min) converted to an exclusively low-affinity state, Rl, and contractile response diminishes. This desensitization process is sensitive to agents that produce inhibition of microfilament actin polymerization (Brenner and Korn, 1979; Insel and Koachman, 1982). If the agonist is promptly removed, the receptor pool returns in 30-60 minutes to a state capable of coupling to Ns and initiating a contractile response to agonist. These receptors recovered from desensitization are fully functional. Recovery (resensitization) from the desensitized state also appears to be dependent on the function of microfilaments. Because IPIN and CGP12177 identify the same pool of receptors under control conditions, it is likely that there is no substantial pool of intracellular receptors prior to agonist exposure.

With prolonged agonist exposure (hours), receptors become no longer accessible to IPIN in a process modulated by microtubules; they are probably internalized (Strulovici et al., 1983). From the evidence reported here, one cannot be certain if receptors are degraded or if their primary structure is conserved, but they are sequestered from IPIN binding. After agonist is removed, re-expression of the receptors in the cell membrane is slow (24-72 hours) and requires protein synthesis.

### Relation of These Findings in Cardiac Tissue to Studies on β-Receptor Regulation in Model Systems

β-Adrenergic receptor regulation has been extensively studied in a variety of model systems with several different mechanisms of receptor regulation appearing to predominate, depending on the tissue studied, as well as other factors (Stadel et al., 1980; Tolkovsky and Levitzki, 1981; Doss et al., 1981; Harden, 1983). Depending on the model system, there are one or two stages of receptor regulation, with or without receptor loss. If receptors are lost, receptor recovery may or may not depend on protein synthesis. Little is known about the integrity of the entire receptor-effector physiological response system at various stages of desensitization and recovery. In most cases, the most distal event studied has been adenylate cyclase activation.

The model system that has been extensively studied and bears greatest similarity to the findings reported here for cultured chick ventricular cells is one of several strains of the C62B glioma cell line studied by Frederich et al. (1983). For one specific strain, it was found that agonist exposure (1 μM isoproterenol) produced a decrease in the number of β-receptors to 40% of control by 4 hours of

### Table 4

<table>
<thead>
<tr>
<th>Growth conditions*</th>
<th>Cells/well</th>
<th>Cells/mg protein</th>
<th>Phenylalanine incorporation (nmol/mg protein per hr)</th>
<th>Thymidine incorporation (counts/min/mg per hr)</th>
<th>Phenylalanine thymidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth in serum 48 hr</td>
<td>7.1 x 10^5</td>
<td>6.4 x 10^5</td>
<td>56</td>
<td>30 x 10^3</td>
<td>1.9 x 10^-3</td>
</tr>
<tr>
<td>Growth is serum 48 hr then serum-free 72 hr</td>
<td>9.5 x 10^5</td>
<td>5.7 x 10^5</td>
<td>45</td>
<td>18 x 10^3</td>
<td>2.5 x 10^-3</td>
</tr>
<tr>
<td>Growth in serum 48 hr, then serum-free 72 hr with 1 X 10^-4 cycloheximide</td>
<td>5.1 x 10^5</td>
<td>4.0 x 10^5</td>
<td>5.6</td>
<td>5 x 10^3</td>
<td>1.2 x 10^-3</td>
</tr>
</tbody>
</table>

* Media in which cultures were grown (serum-containing or serum-free medium).
exposure, findings very similar to those reported here. If cells were exposed to 1 μM isoproterenol for 30 minutes or less, there was no receptor loss, but there was uncoupling of the receptor from adenylate cyclase activation. Furthermore, adenylate cyclase activation recovered in 45 minutes after agonist removal. If cells were exposed to isoproterenol for 4 hours, then it took 60 hours in the absence of isoproterenol for the β-receptor number to recover to control levels; this process was sensitive to cycloheximide. These findings are also analogous to some of the results reported here.

The mechanism of β-adrenergic receptor regulation in model systems is highly dependent on the details of the preparation. For instance, Doss et al. (1981) found that protein synthesis was required for recovery of down-regulated β-receptors in a tumor cell line grown to confluence, but not in subconfluent cells. In the cultured chick ventricular cell system utilized in this study, recovery of the total number of β-receptors reflects new receptor synthesis in a relatively stable population of cells. As in adult cardiac tissue, most cells in our preparation are withdrawn from the cell cycle (Clark and Fischman, 1983). Indeed, during 72 hours’ growth in serum-free medium, the number of cells increased by only 23% and the percent myocytes was unchanged.

Because cells in our preparation desensitized by isoproterenol for 30 minutes almost completely recover their physiological contractile response to isoproterenol 30 minutes after removal of the agonist, one may conclude that there is not a longer-lasting defect distal to the receptor-cyclase complex induced by catecholamine exposure.

In membranes of cultured heart cells, we were able to identify two agonist affinity states for the β-receptor. We have also been able to identify high-affinity binding for agonist in intact cells after very brief (15-second) periods of agonist exposure (Marsh et al. 1984). After longer exposure time, high-affinity receptors cannot be identified, presumably because the high-affinity state is present only in catalytic quantities when GTP is available to interact with N, and rapidly return the receptor to the low-affinity state (Pittman and Molinoff, 1983; Toews and Perkins, 1984).

Role of the Cytoskeleton in Receptor Regulation

Involvement of the cytoskeleton in receptor regulation has been investigated in several model systems, most extensively for polypeptide hormone receptors, but also to some degree for non-peptide adenylate cyclase-coupled receptors. The cytochalasins are a group of secondary mold metabolites that have the property of inhibiting polymerization of actin (Brenner and Korn, 1979; Lin and Lin, 1979), a major component of microfilaments usually found in the cortical cytoplasm (Geiger, 1983). The microfilaments have been shown to make stable contact with the cytosolic face of the plasma membrane (Geiger, 1983), where they may interact with N, or the catalytic subunit of cyclase (Insel and Koachman, 1982). There is considerable evidence that mobility of plasma membrane components in eukaryotic cells may be regulated by microfilaments (Lin and Lin, 1979; Jarett and Smith, 1979; Insel and Koachman, 1982; Geiger, 1983). Our observation that both cytochalasins B and D modulate desensitization to a β-adrenergic agonist supports the view that movement of components of the adenylate cyclase system in the cell membrane may be essential for desensitization.

Although the role of microfilaments in regulation of the cardiac β-receptor has not been reported previously, involvement in receptor regulation by microfilaments has been suggested for other systems (Zor et al., 1979; Insel and Koachman, 1982). Examining the frog erythrocyte model of β-receptor desensitization, Chuang (1981) found that pretreatment of cells with methylamine, which inhibits transglutaminase, a cytoskeleton cross-linking enzyme, inhibited receptor loss and internalization. Thus in other model systems, there is evidence that microfilament function might play a role in receptor regulation. One might infer from our data on contractile desensitization and recovery with concomitant shifts of agonist binding in membranes from R, to R, and back to R, that microfilaments play a direct role in alterations of agonist affinity state of the receptor and facilitate transient sequestration of receptor away from N, (Waldo et al. 1983; Strader et al. 1984). However, data available at this time do not permit one to conclude that microfilaments have a direct role in mediation of the affinity shift, although it is an attractive hypothesis.

The lack of colchicine sensitivity (Table 2) indicates that microtubules are not involved in the initial stage of agonist regulation of the β-receptor in cultured heart cells. Microtubules, which often have a radial arrangement in the cytoplasm, do appear to modulate receptor loss from the pool identifiable by IPIN in our system. A role for microtubules in receptor internalization and/or degradation is thus suggested by our data, and is in concert with the findings of Limas and Limas (1984).

Relation of Receptor Regulation to Cardiac Function

The development of β-receptor assays that permit identification of receptors on intact cells under physiological conditions and the identification of two affinity states for agonist in cell membranes, combined with observations on physiological responsiveness, provide information about localization of receptors and their functional state that has not been previously available for cardiac tissue. Cardiac β-adrenergic receptors, when exposed to agonist, undergo two stages of dynamic regulation: rapid initial desensitization that appears to require micro-
filament function, followed by microtubule-modulated down-regulation or loss of receptors. Recovery of the receptors is relatively slow and requires protein synthesis.

We have demonstrated that exposure of the β-receptor to agonist affects not only receptor-binding properties, but also the physiological expression of agonist interaction—in this case, augmentation of the inotropic state. Other hormones and neurotransmitters such as acetylcholine, thyroid hormone, and cortisol probably have important modulating effects on the β-receptor in addition to those of β-adrenergic agonists. Further investigation will be necessary to delineate these effects, as well as the mechanisms by which important pathophysiological insults, such as ischemia, modulate β-receptor function and physiological expression of the β-receptor properties.

The excellent technical assistance of Nancy Stec and James Greeney is acknowledged. We are grateful to Dr. Peter Libby for helpful advice and for access to data on serum-free culture media in advance of publication, and to Dr. Thomas Smith for many fruitful discussions during the course of these studies.

Supported in part by Grant 81-695 from the American Heart Association with funds contributed in part by the Northeast Chapter, Massachusetts affiliate of the American Heart Association, and by Grants HL00691 and HL18003 from the National Heart, Lung, and Blood Institute. Dr. Marsh is the recipient of a Clinical Investigator Award from the National Institutes of Health.

Address for reprints: James D. Marsh, M.D., Cardiovascular Division, Brigham & Women’s Hospital, 75 Francis Street, Boston, Massachusetts 02115.

Received June 25, 1984; accepted for publication April 11, 1985.

References


Stadel JM, Strulovich B, Nambi P, Lavin TN, Briggs MM, Caron

Circulation Research/Vol. 57, No. 1, July 1985


Stadel JM, Strulovich B, Nambi P, Lavin TN, Briggs MM, Caron
Marsh et al. /Mechanisms of β-Receptor Regulation

Tolkovsky AM, Levitzki A (1981) Theories and predictions of models describing sequential interaction between the receptor, the GTP regulatory unit, and the catalytic unit of hormone dependent adenylate cyclases. J Cyclic Nucleotide Res 7: 139-150

INDEX TERMS: β-Receptor • Cultured heart cell • Desensitization • Cytoskeleton
J D Marsh, D Lachance and D Kim

doi: 10.1161/01.RES.57.1.171

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1985 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/57/1/171

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/