Rapid Recovery of Cardiac β-Adrenergic Receptors after Isoproterenol-Induced “Down”-Regulation

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SUMMARY. Desensitization of β-receptor-linked adenylate cyclase occurs after prolonged occupancy of the β-receptors by their agonists. We have followed the development and recovery from “down”-regulation of β-receptors in enzymatically dissociated cardiac myocytes by using the hydrophilic antagonist [3H]-CGP-12177 to identify surface-bound β-receptors. After in vitro incubation with (-)-isoproterenol, almost 50% of the β-receptors are lost within 10 minutes. Isoproterenol-mediated cyclic adenosine monophosphate accumulation by isolated myocytes was also decreased after a 15-minute preincubation with isoproterenol. ‘Lost’ β-receptors can, however, be recovered when isoproterenol-pretreated, washed cardiac myocytes are incubated at 37°C, 85 ± 7% of the lost β-receptors have returned to the cell surface after 20 minutes of incubation. The requirements for such recycling were investigated. Receptor recovery does not depend on de novo protein synthesis, since it is unaffected by prior exposure to cycloheximide. It is, however, dependent on cellular energy, because it is prevented by adenosine triphosphate depletion and involves a lysosomal step since it is inhibited by the lysosomotropic agent, chloroquine. In addition, the Golgi apparatus and the microtubules are involved in the β-receptor recycling to the cell surface, as evidenced by the inhibitory effects of monensin and colchicine, respectively. The mechanism of isoproterenol-induced down-regulation of cardiac β-receptors involves a rapid, reversible cycling to and from the cytosol and the cell membrane. This intracellular receptor traffic is energy dependent, requires several structures, including lysosomes and microtubules, and may be modified by pathological processes involving the heart. (Circ Res 55: 524-531, 1984)

IT has been known for some time that prolonged occupancy of the β-receptors by their agonists leads to desensitization, i.e., diminished magnitude of agonist-mediated effects. This desensitization is characterized by a decline in the number of β-receptors and/or isoproterenol-stimulated adenylate cyclase activity (Mickey et al., 1975; Harden et al., 1979; Homburger et al., 1980). The process of receptor desensitization is important in cardiac pathophysiology for two reasons. First, many models of experimental or clinical hypertrophy and failure are associated with decreased number of cardiac β-receptors and/or adenylate cyclase activities (Limas and Limas, 1978; Tse et al., 1979; Bristow et al., 1982) with consequent restriction in cardiac inotropic reserve. Second, the usefulness of β-agonists for the treatment of heart failure may be limited through their induction of desensitization (Colucci et al., 1981). Indeed, since the clinical syndrome of heart failure is commonly associated with a substantial activation of the sympathetic nervous system reflected in increased circulating catecholamine levels (Thomas and Marks, 1978), changes in the β-receptor-cyclase system of the diseased myocardium may be, in part, secondary to the desensitization process. Considerable progress has been recently made in elucidating the molecular basis of β-receptor desensitization. The evidence in both nonmuscle and cardiac cells indicates that binding of the agonist to the receptor leads to the eventual internalization of the latter in the cytosol dissociated from the other components of the adenylate cyclase complex (Chuang and Costa, 1979; Stadel et al., 1983; Limas and Limas, 1984). In the frog erythrocyte, the internalized β-receptor is sequestered in a vesicular structure (Stadel et al., 1983) devoid of plasma membrane markers, which may be analogous to the endosome (or receptosome) described for other receptors (Brown et al., 1983). Whether cardiac β-receptors en route to the cytosol during desensitization are similarly sequestered has not been established. More important, it is not known whether internalized cardiac β-receptors are degraded within the cell, or are returned to the cell surface, and what factors regulate this recycling. The recovery of cardiac β-receptors from agonist-induced apparent loss (down-regulation) is the focus of the present report.

Methods

Experiments were carried out on adult male Sprague-Dawley rats weighing 250–350 g. Cardiac myocytes were prepared by enzymatic dissociation using a procedure previously described (Limas, 1982). Briefly, rat ventricles were finely minced and washed free of blood with cold (4°C) dissociation buffer containing (g/liter): NaCl, 6.8; KCl, 0.4; NaH2PO4, 0.06; Na2HPO4, 0.21; and glucose,
Cardiac β-receptors were routinely identified using the hydrophilic ligand (±) [3H]-CGP-12177 [4-[3-(tertiary butylamino-2-hydroxypropoxy)-benzimidazol-2-0n hydrochloride] which labels specifically cell surface-bound receptors (Staehelin and Hertel, 1983; Staehelin et al., 1983). Myocyte suspensions in 0.25 M sucrose-50 mM Tris-HCl (pH 7.4)-10 mM MgCl₂ at 37°C for 2-20 minutes in the presence of (-)-isoproterenol (10⁻⁸ to 10⁻⁵ M) was used as the ligand (10 nM final concentration), the GF/C filters were dried before radioactivity counting.

In order to study isoproterenol-induced down regulation of cardiac β-receptors, we first incubated suspensions of myocytes in 0.25 M sucrose-50 mM Tris-HCl (pH 7.4)-10 mM MgCl₂ at 37°C for 2-20 minutes in the presence of (-)-isoproterenol (10⁻⁸ to 10⁻⁵ M) in a total volume of 0.5 ml. At the end of the incubation, a 10-fold excess of ice-cold Tris-MgCl₂ buffer was added to each tube, and the samples were filtered through Whatman GF/C filters. The filters were washed three times with 5 ml of ice-cold buffer and were counted in 10 ml of Aquasol-2 (New England Nuclear Co.).

FIGURE 1. Affinity and saturability of specific [3H]-CGP-12177 binding sites on cardiac myocytes. Each point represents the mean of triplicate determinations from three separate experiments.

In some experiments, isoproterenol-mediated generation of cAMP by cardiac myocytes was studied. Myocytes were first incubated with or without 1 μM (-)-isoproterenol at 37°C for 15 minutes, washed with excess buffer, and resuspended in 0.25 M sucrose-50 mM Tris-HCl (pH 7.4). Incubation with 10⁻⁸ to 10⁻⁵ M (-)-isoproterenol was carried out at 37°C for 10 minutes in the presence of 0.2 mM 3-isobutyl-1-methylxanthine. Reactions were stopped by adding 2 ml of cold 7% trichloroacetic acid (TCA), followed by centrifugation at 2000 g for 10 minutes. After extraction with ether, the amount of cAMP in the supernatant was determined with the protein-binding assay (Gilman, 1974).

For studies of β-receptor reappearance following isoproterenol-induced down-regulation, myocytes were first incubated with 1 μM (-)-isoproterenol at 37°C for 20 minutes, the incubation was terminated with the addition of excess cold buffer, and the cells were washed twice by centrifuging at 1000 g for 10 minutes. The reaction was terminated with excess cold 0.25 M sucrose-50 mM Tris-HCl (pH 7.4)-10 mM MgCl₂ buffer, and the myocytes were washed extensively before being resuspended in the sucrose-Tris-Mg⁺ buffer. Samples were either used directly for determination of cardiac β-adrenergic receptors (○) or were first lysed (△). The number of β-receptors was determined either at 4°C for 16 hours in the presence of 6 nM [3H]-CGP-12177 (○) or at 37°C for 30 minutes with 6 nM [3H]-CGP-12177 (△). Results are expressed as percent of control (no isoproterenol in the preincubation stage), and represent the mean ± SE for four to six experiments.

FIGURE 2. Time course of β-receptor down-regulation by isoproterenol. Myocyte suspensions (0.4-0.6 mg protein) were preincubated with or without 1 μM (-)-isoproterenol at 37°C for the indicated length of time. The reaction was terminated with excess cold 0.25 M sucrose-50 mM Tris-HCl (pH 7.4)-10 mM MgCl₂ buffer, and the myocytes were washed extensively before being resuspended in the sucrose-Tris-Mg⁺ buffer. Samples were either used directly for determination of cardiac β-adrenergic receptors (○) or were first lysed (△). The number of β-receptors was determined either at 4°C for 16 hours in the presence of 6 nM [3H]-CGP-12177 (○) or at 37°C for 30 minutes with 6 nM [3H]-CGP-12177 (△). Results are expressed as percent of control (no isoproterenol in the preincubation stage), and represent the mean ± SE for four to six experiments.
end of each incubation period, test tubes were transferred to an ice-water bath, and incubation was continued for 16 hours in the cold prior to filtration. In some experiments, myocytes were incubated with substances such as colchicine, trimethylcolchicinic acid, chloroquine, antimycin, 2-deoxyglucose, and monensin, thought likely to interfere with receptor recovery following isoproterenol exposure. The details of these experiments are given in Results. To study the importance of cell integrity for β-receptor down-regulation and reappearance, myocytes preincubated with isoproterenol were either processed as above or resuspended in 10 mM Tris-HCl (pH 7.4)-10 mM MgCl₂ and left on ice for 30 minutes to induce lysis. They were then pelleted and resuspended in 0.25 M sucrose-50 mM Tris-HCl-10 mM MgCl₂ before incubation at 37°C to identify β-receptors.

Results

[³H]-CGP-12177 binds to isolated cardiac myocytes in a saturable manner (Fig. 1A), and Scatchard plots of the binding data reveal a single class of sites with a Kᵦ of 1.4 nM and about 115 fmol/10⁶ cells (Fig. 1B) for an average of 4.5 mg protein/10⁶ cells. The binding shows the expected stereospecificity for β-adrenergic receptors with the (−)-isomers of β-agonists or antagonists being at least two orders of magnitude more potent than the (+)-isomers in competing for [³H]-CGP-12177 sites (data not shown).

Incubation of intact myocytes with (−)-isoproterenol and at 37°C leads to a rapid decline in the number of the assayable β-receptors (Fig. 2), which reaches a plateau of about 50% at 10 minutes of incubation. This decline is not seen if cells are lysed first and [³H]-CGP-12177 binding is then assayed. A similar apparent "loss" of β-receptors can be shown if the lipophilic ligand [³H]-DHA is used provided that [³H]-DHA binding is assessed at 4°C. That one does not deal with a true β-receptor loss is indicated by the unchanged [³H]-DHA binding sites in lysed cells after isoproterenol. The extent of cell-surface β-receptor "loss" after isoproterenol preincubation shows a steep dependence on the isoproterenol concentration (Fig. 3) with half maximal inhibition at 0.5 μM. Furthermore, there is a change in the numbers, but not the affinity, of the β-receptors after isoproterenol incubation (Fig. 4). To demonstrate the loss of surface receptors after preincubation at 37°C in the presence of isoproterenol, we incubated washed and resuspended cells at 4°C for 16 hours (the time adequate for equilibration). Assay at 37°C does not demonstrate down-regulation (Fig. 2), probably because of the rapid recycling of the β-receptors (Staehelin et al., 1983). The decline in β-receptor numbers after isoproterenol was
not due to persistent occupancy of the receptors at 4°C. After incubation of myocyte suspensions with or without 10 μM (-)-isoproterenol at 4°C for 30 minutes, washing, and further incubation with [3H]-CGP-12177 at 4°C for 16 hours, no difference was seen between control and isoproterenol-treated cells. When, however, [3H]-CGP-12177 binding was assayed after isoproterenol incubation at 4°C without washing the cells, a substantial reduction in binding was found, as expected. Isoproterenol-induced down-regulation, therefore, does not occur at 4°C, and the loss of [3H]-CGP-12177 binding sites is not secondary to displacement by isoproterenol.

In addition to a concentration-dependent decline in assayable cardiac β-receptors following isoproterenol, there is a substantial loss in the isoproterenol-induced stimulation of cAMP production by isolated cardiac myocytes which have been preincubated with isoproterenol (Fig. 5), suggesting true desensitization of the receptor-cyclase system. These results do not support the presence of substantial numbers of "spare" β-receptors. We then proceeded to examine the reappearance of β-receptors after isoproterenol-induced down-regulation. After 20-minutes of incubation with 1 μM (-)-isoproterenol at 37°C, the cells were washed extensively with sucrose-Tris-Mg++ buffer to remove unbound agonist. [3H]-CGP-12177 was added, and the suspension was incubated at 37°C for variable lengths of time. As shown in Figure 6, there is a rapid reappearance of β-receptors at 37°C. In 11 consecutive experiments, at the end of preincubation the number of β-receptors had declined to 56 ± 7% of controls (incubated in the absence of isoproterenol) and, at 20 minutes, had been restored to 85 ± 6% of the control value. The extent of receptor recovery varies with each batch of myocardial cell preparation and ranges from 74 to 100%. A similar "recovery" is identified when incubation of washed, isoproterenol-treated cells is carried out at 37°C in the absence of ligand and [3H]-CGP-12177 is added in the cold. The importance of having intact cells is demonstrated by the fact that no recovery can take place if the cells are

![Graph](image-url)
lysed after exposure to isoproterenol. The affinity of the receptors for [3H]-CGP-12177 assessed from Scatchard plots of binding data is unaltered both during down-regulation and after recovery.

The mechanism of receptor recovery after down-regulation was then explored. Such recovery does not appear to depend on de novo synthesis, since cycloheximide preincubation, which results in 90% inhibition of [3H] leucine incorporation into myocardial cells, has no effect on the rate or extent of β-receptor reappearance (Fig. 7).

To study the influence of ATP levels on receptor recovery, we preincubated cells with antimycin and deoxyglucose at concentrations that were effective in lowering cellular ATP levels [determined according to Strehler (1974) from 2.1 ± 0.1 mM in controls to 0.6 ± 0.2 mM in treated cells, P < 0.01]. Such ATP depletion did not affect isoproterenol-induced down-regulation, but essentially prevented receptor recovery after isoproterenol (Fig. 8). In addition to its energy dependence, the recovery process requires a lysosomal step, since preincubation with the lysosomotropic agent, chloroquine, completely prevented the reappearance of the receptor (Fig. 9). Also, there is a definite dependence on intact microtubule assembly, since colchicine, but not its analog, trimethylcolchicinic acid, which does not bind to tubulin, effectively blocked the reappearance of the receptors (Fig. 10). Vinblasrine has the same effect, whereas lumicolchicine is ineffective (data not shown). Finally, the carboxylic ionophore monensin completely blocks β-receptor recycling while having no effect on isoproterenol down-regulation. In six experiments, isoproterenol-treated myocytes had 65 ± 4% of β-receptors in control cells compared to 63 ± 5% in myocytes exposed to 50 µM monensin in addition to isoproterenol (1 µM). After 20 minutes of incubation of washed myocytes at 37°C, the number of β-receptors recovered to 90 ± 8% of controls in the absence of monensin, whereas it was unchanged (65 ± 4%) of controls in monensin-treated myocytes.

**Discussion**

Despite its significance for cardiac pathophysiology, relatively little is known about the regulation of β-receptor desensitization in cardiac myocytes. Recently, we presented evidence that, after a single injection of isoproterenol in rats, cardiac β-receptors are redistributed from the cell membrane to the cytosol (Limas and Limas, 1983). This internalization depends on intact microtubule assembly, since it is prevented by inhibitors of tubulin polymerization such as colchicine and vinblastine, but not by lumi-
The fate of the internalized β-receptor has not been established. Studies by Strulovici et al. (1983) in frog erythrocytes, using photoaffinity labeling, suggest that no significant degradation occurs and that almost all the receptors can be accounted for by the combination of plasma membrane- and cytosol-associated receptors. This contrasts with the low recovery of intracellular β-receptors after isoproterenol reported by others (Chuang and Costa, 1979) in frog erythrocytes, and explained on the basis of lysosomal degradation. Studies in other cell systems utilizing different types of receptors have suggested that the majority of internalized receptors are eventually reincorporated into the plasma membrane (Doss et al., 1981).

Several features of the recycling process have emerged from this study. First, it does not appear to require de novo synthesis of the receptors, since it is not inhibited by cycloheximide. This is consistent with the rapidity of the recycling process, and is in agreement with observations in other cell types that recovery of membrane receptors occurs in cells that synthesize very little new protein (Mukherjee et al., 1976), or even after protein synthesis is inhibited (Doss et al., 1981; Hertel and Staehelin, 1983). Second, recovery of internalized cardiac β-receptors is an energy-requiring process and is prevented by metabolic inhibitors. This ATP dependence probably is related to the structural compartmentalization of the internalized receptors. According to current concepts (Brown et al., 1983), receptors are translocated to the cytoplasm within specialized structures (coated vesicles) which rapidly shed their clathrin coats and fuse with one another to form larger vesicles, called endosomes (or recep-tosomes). The requirement for ATP may reflect the presence of an ATP-driven protein pump in the endosomes similar to that previously described in the lysosomes (Schneider et al., 1981). Alternatively, ATP may be required for uncoating of the endosomes (Geisow, 1982).

An important aspect of the recycling of internalized cardiac β-receptors is its sensitivity to chloroquine, which is known to inhibit lysosomal function by raising intralysosomal pH (Ohkuma and Poole, 1978). Therefore, chloroquine inhibition of β-receptor recycling (shared by IgG, transferrin, and mannose-glycoconjugate receptors) (Tolleshaug and Berg, 1979; Gonzalez-Noriega et al., 1980; Tietze et al., 1980; Hertel and Staehelin, 1983) may indicate a lysosomal phase in the pathway back to plasma membrane. An alternative regulatory site may be the endosome itself, since it is known that the acid pH within the endosome is needed to dissociate the ligand-receptor complex, a prerequisite for recycling (Geisow, 1982). Therefore, our results are compatible with alkalization of either the endosome or lysosome. Different receptors utilize lysosomes to different extents in their shuttling back to the cell surface. The short loop, excluding the lysosome, is probably predominantly used by very rapidly cycling receptors (e.g., the LDL receptor) (Brown et al., 1983).
ery is compatible with either mechanism, but, perhaps, more on the scale of a lysosome-inclusive pathway.

Two more structures involved in the recovery process are the Golgi apparatus and microtubules. Suggestive evidence for the first comes from the inhibitory effects of monensin, a tricarboxylic antibiotic which exchanges monovalent cations across cell membranes (Pressman, 1976). Such ion gradients are thought to be necessary for budding of Golgi membranes (Tartakoff and Vassalli, 1978) which is an integral part of the recycling pathway (Basu et al., 1981). The microtubule dependence is indirectly deduced from the inhibitory effects of colchicine (but not its inactive analog, trimethylolchicinic acid) added during the recovery phase. This observation is in accordance with the report (Staeelin and Hertel, 1983) that the Ca++ ionophore A23177 prevents β-receptor recycling in CG glioma cells (an effect which we have confirmed in myocardial cells), since calcium is known to depolymerize microtubules.

The integrity of the recovery phase of receptor cycling is very important for maintaining the normal complement of membrane-bound receptors, the only alternative mechanism being de novo synthesis. Since the turnover rate of cardiac β-receptors is normally slow (Pitha et al., 1982), de novo synthesis may not be utilized under physiological conditions. Pathological states, however, may interfere with the adequacy of receptor recycling through several mechanisms involving the energetics of the cell, microtubular assembly, lysosomal integrity, ionic gradients, etc. We have already some evidence that the internalization phase of the desensitization process may be altered in hypertrophy (Limas and Limas, 1984). Whether the recycling pathways can also be affected is currently under study in our laboratory.

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