Certain $\beta$-Blockers Can Decrease $\beta$-Adrenergic Receptor Number: II. Down-Regulation of Receptor Number by Alprenolol and Propranolol in Cultured Lymphoma and Muscle Cells

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We have used two different cultured cell lines—S49 lymphoma cells and BC3H-1 muscle cells—to examine the regulation of $\beta$-adrenergic receptors by receptor antagonists. Rather than an increase ("up-regulation") of receptor number that such antagonists often produce, we found that certain $\beta$-blockers elicit a decrease ("down-regulation") of beta-adrenergic receptors. Alprenolol and propranolol, but not sotalol or ICI 118,551, at concentrations of 10–100 nM down-regulated $\beta$-adrenergic receptors 20–70% following 16–20 hours of treatment of S49 or BC3H-1 cells. Several observations suggest that this phenomenon depends upon beta-receptor interaction, including stereoselectivity [(−)-enantiomers more potent than (+)-enantiomers], blockade of the effect by ICI 118,551, absence of down-regulation of $\alpha$-adrenergic receptors in BC3H-1 cells, and lack of a decrease in beta-adrenergic receptor-independent (forskolin-stimulated) cyclic AMP accumulation in S49 cells. The possibility of retained antagonist interfering with receptor measurement was precluded by the fact that the antagonist-induced decrease in receptor number required several hours incubation and occurred without a prominent change in receptor affinity. The ability of the $\beta$-blockers to elicit down-regulation did not correlate with hydrophobicity of the drugs. Antagonist-induced down regulation of beta-adrenergic receptors did not occur in S49 lymphoma cells that lack the $\alpha$-subunit of $G_s$, the guanine nucleotide-binding regulatory protein, thus implying a requirement for receptor-$\alpha$ interaction in eliciting $\beta$-receptor down-regulation. The ability of certain antagonists to promote a down-regulation of $\beta$-adrenergic receptors provides a mechanism that may contribute to the pharmacological activity of these agents. (Circulation Research 1988;63:279–285)

The accompanying manuscript by DeBlasi et al documents a similar or identical phenomenon of $\beta$-blocker-induced receptor down-regulation elicited by two drugs, alprenolol and propranolol.

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Prolonged exposure of target cells to agonists that stimulate receptor-mediated responses frequently results in a loss of the corresponding receptor and its associated responses. These phenomena are termed "down-regulation" and "desensitization," respectively. $\beta$-Adrenergic receptors are perhaps the most well-studied receptors for which these phenomena have been observed.\textsuperscript{1–4} Receptor antagonists, on the other hand, elicit no response by themselves, but they do block agonist-mediated responses and agonist-promoted desensitization and down-regulation. Treatment of animals or people with receptor antagonists, including $\beta$-adrenergic receptor antagonists ($\beta$-blockers), often increases ("up-regulates") receptor number, perhaps as a consequence of blocking tonic down-regulation induced by ambient levels of agonist.\textsuperscript{5–9} When administered at low concentrations, antagonists are generally believed to have no effect other than competitive blockade of agonist occupation of the receptor. As described herein, we and others\textsuperscript{10–12} have recently obtained results that do not conform to this axiom. Certain $\beta$-blockers can be shown to decrease $\beta$-adrenergic receptor number. In this report, we document some of the characteristics of this phenomenon of $\beta$-blocker–induced receptor down-regulation elicited by two drugs, alprenolol and propranolol.
as expressed by acute exposure of cells to two different β-blockers, bopindolol and tertatolol.

Materials and Methods

Materials

The following drugs were received as generous gifts: phentolamine mesylate from CIBA-Geigy Corporation, Summit, New Jersey; (-)-cyanopindolol (CYP) from Dr. G. Engel, Sandoz Pharmaceuticals, East Hanover, New Jersey; (-)-propranolol and (+)-propranolol from Ayerst Research Laboratories, New York. Octanol was purchased from Sigma Chemical, St. Louis, Missouri. Carrier-free Na[123I] (>350 mCi/ml) and cyclic [2,8-3H]AMP (30 Ci/mmole) and [3H]prazosin (81 Ci/mmmole) were obtained from Du Pont/New England Nuclear, Boston, Massachusetts. Dulbecco's Modified Eagle's Medium (DMEM) and fetal calf serum were obtained from Grand Island Biological Company, Grand Island, New York, and 0.25% sterile trypsin solution from Irvine Scientific, Santa Ana, California. Flasks and dishes were manufactured by Falcon.

Cell Culture

The BC3H-1 nonfusing muscle cells used in this study have been described previously. The cells were routinely grown in T-flasks in DMEM supplemented with 10% (vol/vol) fetal calf serum and maintained at 37°C in a humidified atmosphere of 10% CO₂ in air. The cells were subcultured at 3-day intervals, using trypsin to dissociate them from the plastic substrate. For experimental purposes, unless indicated otherwise, approximately 1.5×10⁶ cells were seeded into 150-mm diameter culture dishes containing 25 ml of culture medium. From this starting density, the cells reached confluence within 2–4 days. Wild-type and cyc⁻S49 murine lymphoma cells were grown in suspension culture as 2–4 days. Wild-type and cyc⁻S49 murine lymphoma cells were grown in suspension culture as described previously. S49 cells were grown in suspension culture as described previously. S49 cells were used only when growing logarithmically and when viability (determined by exclusion of trypan blue) was ≥80%. cyc⁻S49 Lymphoma cells are cells that possess β-adrenergic receptors but lack the messenger RNA and gene product of the α-subunit of G₁, the stimulatory guanine nucleotide–binding protein.

Preparation of I[125I]ICYP

(-)-CYP was iodinated as described by Engel and coworkers. [125I]ICYP was stored at -20°C for no longer than two months, when it still migrated as a single peak on silica gel thin-layer chromatography with pyridine/glacial acetic acid/water (0.33: 0.6: 0.9: 0.07, vol/vol) as solvent. The [125I]ICYP was assumed to be iodinated to the maximal theoretical specific activity of 2,200 Ci/mmol; correction for decay of [125I]ICYP assumed that the product of radioactive decay was unable to bind to the β-receptor since comparison with [3H]dihydroalpranolol showed this method to yield consistent receptor number in rat lung membranes.

Incubation of Cells With Antagonists

Antagonists were added to the growth medium of BC3H-1 cells that had just reached confluence or to S49 cells at a density of 0.5–0.8×10⁶ cells/ml. After 16–20 hours of incubation, the cells were washed free of antagonist. In the case of BC3H-1 cells, the cells were washed four times at 37°C in situ on the dish with DMEM containing 20 mM, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), pH 7.4, and 1 mg/ml bovine serum albumin (Fraction V from Sigma). S49 cells were washed four times by centrifugation in 37°C DMEM containing 10% horse serum. Radioligand binding experiments were carried out as described below.

Radioligand Binding Assays

A crude membrane preparation of BC3H-1 cells, free of nuclei, was prepared as described previously. The BC3H-1 membranes, suspended in 50 mM Tris-HCl (pH 7.6) and 10 mM MgCl₂, were incubated in a final volume of 0.25 ml for [125I]ICYP binding or 0.5 ml for [3H]prazosin binding studies at a protein concentration of 0.1–0.3 mg/ml. The binding reaction was carried out for 60 minutes at 30°C. Nonspecific binding was always less than 20% of total binding in these experiments. Binding to intact S49 cells was conducted using [125I]ICYP and 1 μM (-)-propranolol to define nonspecific binding, as described previously. In the case of Figures 3 and 6, where receptor density was estimated by measuring binding of a single concentration of [125I]ICYP, quadruplicate measurements were made of both total and nonspecific binding at a single radioligand concentration (0.2 nM). In all other cases, receptor density was determined by means of saturation binding isotherms performed using six concentrations of radioligand. [3H]Prazosin samples were counted in a Beckman LS-330 liquid scintillation spectrometer (Fullerton, California) at 40% efficiency; [125I]ICYP samples were counted in a Packard gamma counter (Downers Grove, Illinois) at 85% efficiency.

Cyclic AMP Accumulation

Cyclic AMP accumulation in intact S49 cells was assessed by incubation of cells with 1 μM forskolin or 1 μM (-)-isoproterenol for 15 minutes, with assay of cellular cyclic AMP levels as described previously.

Data Analysis

For Scatchard analyses of equilibrium binding data, total binding at each concentration of radioligand was determined in triplicate and nonspecific binding in duplicate. Linear regression analyses were carried out for all estimates of nonspecific binding since these varied linearly over the range of radioligand concentrations tested. Specific binding at each concentration of radioligand was then calculated from the difference between the mean of the values for total binding and the value for nonspe-
cific binding computed from that given by linear regression. Data were fit to the relation $B = B_{\text{max}}[L]/(K_d + [L])$, which in turn was used to simulate the saturation binding isotherms shown in the figures. The number of binding sites ($B_{\text{max}}$) and the dissociation constant ($K_d$) were obtained from Scatchard analysis of the data; $[L]$ represents the free radioligand concentration.

All experimental protocols were replicated as indicated in the figure legends and yielded similar results on each execution. Unless stated otherwise, representative data are presented. Individual data points generally varied less than 10% away from the mean. Error bars have been omitted from some figures for the sake of clarity, but where these are shown, they indicate the standard error of the mean.

**Protein Determination**

Protein was determined by the method of Peterson, employing log-log transformation to yield a linear standard curve. Bovine serum albumin, containing sodium azide (1 mg/ml) as a noninterfering bactericide, was employed for standards.

**Results**

**Antagonist Down-Regulation of β$_p$-Adrenergic Receptors**

BC3H-1 muscle and S49 lymphoma cells both contain a pure population of β$_p$-adrenergic receptors. In initial studies, these cell types were incubated for 16–20 hours in growth medium containing concentrations of β-blockers that were 10–200-fold in excess of their dissociation constants ($K_d$) measured in separate experiments (data not shown). β-Adrenergic-receptor concentrations following treatment with antagonists were measured in either membranes prepared from BC3H-1 cells (Figure 1) or in intact S49 cells (Figure 2) using the radiolabeled β-receptor antagonist [I$^{125}$I]ICYP. Scatchard analysis of these data divided the antagonists into two groups: those that had no effect on receptor number (sotalol and ICI118,551) and those that elicited a substantial (20–80%) decrease in [I$^{125}$I]ICYP affinity. The kinetics for the antagonist-mediated decrease in [I$^{125}$I]ICYP affinity. The kinetics for the

![Figure 1](https://example.com/figure1)

**Figure 1.** Antagonist-mediated down-regulation of β$_p$-adrenergic receptors in BC3H-1 muscle cells. BC3H-1 cells were incubated for 16 hours in growth medium with no added drugs (■), 0.1 nM (-)-alprenolol (○), or 90 μM sotalol (△, ○, △). The cells were washed and membranes were prepared to which binding of [I$^{125}$I]ICYP was measured, as described in "Materials and Methods." Scatchard plots of a representative experiment are shown. Pooled data from three experiments yielded control, $K_d=107±10$ pM, $B_{\text{max}}=53±2$ fmol/mg; sotalol, $K_d=109±7$ pM, $B_{\text{max}}=54±4$ fmol/mg; and (-)-alprenolol, $K_d=371±32$ pM (p<0.01), $B_{\text{max}}=20±2$ fmol/mg (p<0.005).

![Figure 2](https://example.com/figure2)

**Figure 2.** Antagonist down-regulation of β$_p$-adrenergic receptors in S49 lymphoma cells. A representative experiment illustrates the effect of long term exposure of S49 cells to various antagonists. S49 cells were incubated for 20 hours in growth medium with no added drugs (■), 23 nM ICI118,551 (○), 5 μM sotalol (△, ○, △), 100 nM alprenolol (●), 15 nM (-)-propranolol (●, ○). The cells were washed and [I$^{125}$I]ICYP binding was measured. Pooled data from three experiments yielded control, $K_d=12±1$ pM, $B_{\text{max}}=1163±60$ sites/cell; sotalol, $K_d=13±2$ pM, $B_{\text{max}}=1157±62$ sites/cell; ICI118,551, $K_d=13±1$ pM, $B_{\text{max}}=1183±75$ sites/cell; propranolol, $K_d=14±1$ pM, $B_{\text{max}}=935±16$ sites/cell (p<0.05); and alprenolol, $K_d=13±2$ pM, $B_{\text{max}}=603±18$ sites/cell (p<0.01).
TABLE 1. Concentration Dependence of (−)-Alprenolol in Eliciting β₂-Receptor Down-Regulation

<table>
<thead>
<tr>
<th>Concentration of (−)-alprenolol</th>
<th>BC3H-1 membranes</th>
<th>S49 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K₂ (pM)</td>
<td>Bₘₜₐₓ (fmol/mg)</td>
</tr>
<tr>
<td>Control</td>
<td>116 ± 11</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>3 nM</td>
<td>247 ± 57</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>10 nM</td>
<td>287 ± 62</td>
<td>40 ± 7</td>
</tr>
<tr>
<td>100 nM</td>
<td>560 ± 51</td>
<td>24 ± 1</td>
</tr>
</tbody>
</table>

BC3H-1 and S49 cells were incubated for 16–20 hours in growth medium at 37°C with the indicated concentrations of β₂-antagonists. After extensive washing, receptor density was measured in membranes prepared from BC3H-1 cells and on intact S49 cells. Results shown are the mean ± SEM of three experiments.

adrenergic receptors measured on S49 cells by [¹²⁵I]ICYP binding was relatively slow, requiring many hours exposure of cells to alprenolol or propranolol (Figure 3).

Specificity of Antagonist-Mediated Down-Regulation of β₂-Adrenergic Receptors

The water/octanol partition coefficients of the four adrenergic antagonists used in this study were measured in order to determine whether the ability of β₂-antagonists to decrease β₂-adrenergic receptor number was related to hydrophobicity of the drugs (Table 2). Since ICI 118,551 was equally as hydrophobic as alprenolol, and yet unlike alprenolol did not elicit receptor loss (Figures 1 and 2), hydrophobicity does not appear likely to be a critical factor in the ability of a β-blocker to elicit the decrease in β-adrenergic receptor number that we describe.

Evidence that receptor occupancy is an absolute requirement for antagonist-mediated down-regulation was provided by several types of experiments. The effect of propranolol to decrease β₂-adrenergic receptor number was expressed in a stereoselective manner. BC3H-1 cells were incubated for 16 hours in growth medium containing 100 nM (+)- or (−)-propranolol. The β₂-adrenergic receptor concentration in membranes prepared from these cells was reduced 20% by 100 nM of the (+)-enantiomer and 60% (p<0.02) by the (−)-enantiomer of propranolol (Figure 4). We also examined the stereoselectivity of the effect of propranolol in studies with S49 cells. S49 cells were incubated for 20 hours in growth medium containing 50 nM (−)- or (+)-propranolol. After the cells were washed to remove the propranolol isomers, we quantitated the number of cellular β-receptors and the ability of 1 μM (−)-isoproterenol to elevate intracellular cyclic AMP. Preincubation of S49 cells with 50 nM (−)-propranolol decreased both β₂-receptor concentration and isoproterenol-stimulated cyclic AMP accumulation 40% more than did the (+)-isomer of propranolol, which itself did not diminish either β-receptor concentration or β-mediated cyclic AMP accumulation (Table 3).

The receptor specificity of this antagonist-mediated loss of β₂-receptors was demonstrated by assessing the effects of antagonists on other related membrane proteins. Incubation of BC3H-1 cells with 100 nM (−)-propranolol which decreased β₂-receptors 60% (Figure 4), elicited a small but not statistically significant change in α₁-adrenergic receptor number (Figure 5). In addition, incubation of S49 cells with 50 nM (−)-propranolol failed to decrease forskolin-stimulated cyclic AMP accumulation, whereas isoproterenol-stimulated cyclic AMP accumulation was markedly decreased under these conditions (Table 3). Thus, we conclude that the loss of re-

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Kinetics of antagonist-mediated down-regulation of β₂-receptors in S49 cells. S49 cells were incubated in growth medium containing 100 nM (−)-alprenolol (•—) or 100 nM (−)-propranolol (□—) for the indicated lengths of time. The cells were washed and receptor density was estimated by measuring binding of 0.2 nM [¹²⁵I]ICYP. The data shown is the mean ± SEM of three experiments.

**Table 2.** Water: Octanol Ratio Partition Coefficients for β₂-Adrenergic Blockers

<table>
<thead>
<tr>
<th>Water/H₂O (nm)</th>
<th>Water:octanol ratio partition coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alprenolol</td>
<td>269.5</td>
</tr>
<tr>
<td>Propranolol</td>
<td>288</td>
</tr>
<tr>
<td>Sotalol</td>
<td>226</td>
</tr>
<tr>
<td>ICI 118,551</td>
<td>278</td>
</tr>
</tbody>
</table>

Partition coefficients were determined by dissolving 100 μM drug in 50 mM sodium phosphate buffer, pH 7.0, and measuring the concentration of drug in the aqueous solution before and after shaking with an equal volume of octanol.
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FIGURE 4. Stereoselectivity of propranolol in eliciting β-receptor down-regulation. BC3H-1 cells were incubated for 16 hours in growth medium with no added drug (O—O), 100 nM (-)-propranolol (*—*), or 100 nM (+)-propranolol (+...+). The cells were washed and membranes were prepared for measurement of binding of [125I]ICYP. The data shown is representative of that obtained in three experiments. Control, Kd=120±7 pM, Bmax=53±6 fmol/mg; (+)-propranolol, Kd=215±10 pM (p<0.005), Bmax=44±6 fmol/mg (p<0.05); and (-)-propranolol, Kd=463±50 pM (p<0.005), Bmax=21±3 fmol/mg (p<0.02).

FIGURE 5. Propranolol does not down-regulate BC3H-1 α1-adrenergic receptors. BC3H-1 cells were incubated for 16 hours in growth medium with no added drug (O—O) or with 100 nM (-)-propranolol (*—*). The cells were washed, membranes were prepared and binding of [3H]prazosin was measured. Data representative of that obtained in three separate experiments is shown. Control, Kd=108±13 pM, Bmax=109±8 fmol/mg; and propranolol, Kd=111±9 pM, Bmax=107±2 fmol/mg.

dose-dependency, several hour kinetics, stereoselectivity [(-)>(+)], specificity for β-receptors, requirement for the α-subunit of the guanine nucleotide-binding regulatory protein, Gα, and a lack of correlation with hydrophobicity of the antagonist.

Discussion

We have found that certain adrenergic antagonists (alprenolol and propranolol), but not others (sotalol and ICI 118,551), decrease the concentrations of β-adrenergic receptors in both BC3H-1 muscle and S49 lymphoma cells. This phenomenon of antagonist-induced decrease in β-receptor concentration displays the following characteristics:

Table 3. Specificity of Antagonist Down-Regulation of β-Adrenergic Receptors in S49 Cells

<table>
<thead>
<tr>
<th>Cyclic AMP accumulation</th>
<th>1 μM Isoproterenol (pmol cAMP/10⁶ cells/15 min)</th>
<th>1 μM Forskolin</th>
<th>[125I]ICYP binding (sites/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>405±35</td>
<td>1,387±129</td>
<td>1,076±97</td>
</tr>
<tr>
<td>(-)-Propranolol (50 nM)</td>
<td>291±12</td>
<td>1,501±46</td>
<td>755±42 (p&lt;0.05)</td>
</tr>
<tr>
<td>(+)-Propranolol (50 nM)</td>
<td>407±37</td>
<td>1,573±128</td>
<td>1,129±20</td>
</tr>
</tbody>
</table>

Cells were incubated for 20 hours in growth medium at 37°C with the indicated concentrations of β-agonists. After extensive washing, cells were examined for either isoproterenol or forskolin-mediated stimulation of intracellular cAMP accumulation, and β-adrenergic receptor levels with 100 pM ICYP. Results shown are the mean ± SEM of three experiments.
that we observed with propranolol and alprenolol appears to require a longer period of incubation with target cells than does the rapidly occurring decrease in receptor number observed with tertatolol and bopindolol. We speculate that certain β-blockers, such as propranolol, have offsetting effects in vivo, that is, a decrease in β-adrenergic receptor number in the manner we have described and an increase in β-adrenergic receptor number caused by blocking a tonic down-regulation of receptors produced by neuronal and circulatory catecholamines. If this hypothesis is correct, then sotalol or ICI 118,551 should elicit a greater up-regulation in β-receptor number in vivo than does either propranolol or alprenolol.

The mechanism whereby propranolol and alprenolol decrease β-receptor number is not known. One possible mechanism is through long-lived occupation of the receptor. However, the calculated dissociation rate constant for (−)-propranolol on S49 cells (1.0/min at 37°C) corresponds to a half-life of receptor occupancy of 0.7 minute, a time that would be incompatible with long-lived occupation of the receptor by this drug. Since effective concentrations of β-blockers that elicit decreases in β-adrenergic receptor number are many times greater than those that cause half-maximal occupation of receptors, this disparity in effective concentrations argues against a receptor-mediated mechanism. However, other observations, such as stereoselectivity, blockade of down-regulation by ICI 118,551, requirement for the guanine nucleotide–binding protein (G), failure to alter α₁-adrenergic receptor concentration and effects on cyclic AMP accumulation argue in favor of a receptor-mediated mechanism. Neither alprenolol nor propranolol, in the 1–100 nM range, measurably elevates intracellular cyclic AMP levels in S49 cells (data not shown), making it unlikely that the down-regulation is mediated by elevated levels of cyclic AMP in these cells.

Propranolol and alprenolol are known to exhibit "membrane stabilizing activity." Such activity comprises many diverse effects, including local anesthetic-like properties, ability to protect red blood cells from hemolysis, and inhibition of serotonin uptake and release by platelets, and appears to be related to the ability of some β-blockers to perturb membrane structure. However, all such effects have been reported to require antagonist concentrations of at least micromolar concentrations and are not stereoselective. In contrast, the decrease in receptor number that we observed following overnight incubation of cells with propranolol and alprenolol requires concentrations of these drugs in the 1–100 nM range, exhibits stereoselectivity, and is prevented by other β-blockers that do not exhibit this effect. The requirement for functional G for the antagonist-induced β-adrenergic receptor down-regulation is perhaps the most compelling evidence for a specific mechanism for this phenomenon. How this requirement for G contributes to antagonist-induced receptor loss is unknown. Nevertheless, it does point to some kind of functional interaction between the receptor and G, driven by antagonist, that causes the receptor loss. This in turn suggests that certain antagonists may be able to induce a conformational change in receptors that in some way mimics that induced by agonists. This action would suggest that antagonists are able to drive some of the responses to receptor occupation that have hitherto been ascribed solely to agonists.

In contrast to our observations, Reynolds and Molinoff observed no down-regulation of β-adrenergic receptors in S49 cells by propranolol, which in their hands was able to prevent the down-regulation induced by another β-blocker, pindolol. We are unable to explain this discrepancy between our results and those of Reynolds and Molinoff, although differences in batches of cells, sera, or other technical considerations may have contributed to the different results.

The concentrations of alprenolol and propranolol that we found to be effective in decreasing β-adrenergic receptor number are similar to those achieved clinically in patients receiving these drugs. Thus, it is conceivable that some β-adrenergic receptor antagonists can alter response to endogenous catecholamines by at least two mechanisms: a rapidly appearing competitive blockade and a decrease in β-adrenergic receptor number that we describe here. It should prove of interest to assess effects of other β-adrenergic antagonists and other target cells for the frequency with which this latter phenomenon occurs. Depending on the particular β-blocker, this decrease in β-adrenergic receptor number may be nonexistent, rapid or slowly appearing, as described in this report.
References


Key Words • beta-receptors • alprenolol • propranolol
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