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Down Regulation of *Beta* Adrenergic Receptors in S49 Lymphoma Cells Induced by Atypical Agonists[1]

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ABSTRACT

The ability of the atypical agonists celiprolol and pindolol to induce sequestration and down regulation of *beta* adrenergic receptors was investigated in S49 lymphoma cells. Sequestration was measured as the loss of binding sites for [^{3}H]CGP-12177, a hydrophilic radioligand that binds only to surface *beta* adrenergic receptors at 6°C. Down regulation was measured as the loss of binding sites for [^{125}I]iodopindolol, a lipophilic radioligand which at 37°C binds to both surface and sequestered receptors. Pindolol and celiprolol do not stimulate adenylate cyclase in membranes from wild-type (WT) S49 cells or do they induce the sequestration of *beta* adrenergic receptors on intact cells. Incubation of WT S49 lymphoma cells with isoproterenol for 24 hr resulted in the loss of 75% of total cellular *beta* adrenergic receptors (down regulation). Exposure of WT S49 cells to pindolol or celiprolol for 24 hr resulted in the loss of approximately half of the total cellular *beta* adrenergic receptors. In mutant S49 cells [cyc$^-$ (variant of S49 lymphoma cells which lacks N_s activity) and UNC (variant of S49 lymphoma cells in which N_s is present but cannot interact with *beta* adrenergic receptors)] in which interaction of *beta* adrenergic receptors with the stimulatory guanine nucleotide binding regulatory protein (N_s) does not occur, a 24-hr incubation with isoproterenol caused the loss of approximately half of the *beta* adrenergic receptors, whereas pindolol and celiprolol caused no change in the number of receptors. These results suggest that there are two mechanisms of down regulation of *beta* adrenergic receptors in S49 cells. One mechanism does not involve interaction of *beta* adrenergic receptors with N_s but does require sequestration of receptors. The other mechanism requires interaction of *beta* adrenergic receptors with N_s but does not involve sequestration of receptors. Pindolol and celiprolol appear to induce selectively the latter mechanism of down regulation of *beta* adrenergic receptors.

Occupancy of *beta* adrenergic receptors by agonists leads to activation of the enzyme adenylate cyclase, resulting in an increase in cellular levels of cyclic AMP. In the continued presence of an agonist, adenylate cyclase becomes refractory to further stimulation by catecholamines by a process referred to as tachyphylaxis or desensitization (Harden, 1983; Sibley and Lefkowitz, 1985). The molecular mechanisms involved in agonist-induced desensitization of this system are not well understood. The initial step appears to involve an alteration in the *beta* adrenergic receptor such that it cannot interact with the other components of the adenylate cyclase system (Su *et al.*, 1980; Homburger *et al.*, 1980; Green and Clark, 1981). Concomitant with this, most of the surface *beta* adrenergic receptors rapidly become sequestered or internalized away from the plasma membrane (Chuang and Costa, 1979; Harden *et al.*, 1980; Stadel *et al.*, 1983). These sequestered receptors are accessible to lipophilic ligands but inaccessible to hydrophilic ligands, and this property has been exploited to characterize the process of sequestration (Staehelin *et al.*, 1983; Toews and Perkins, 1984). For example, [^{3}H]CGP-12177 is a hydrophilic antagonist at *beta* adrenergic receptors that binds preferentially to surface receptors on intact cells (Staehelin and Hertel, 1983). This radioligand has been used to quantitate the number of surface *beta* adrenergic receptors remaining after agonist-induced sequestration (Hertel *et al.*, 1983a, b).

After prolonged exposure to an agonist, the process of down regulation of *beta* adrenergic receptors occurs in mammalian cells (Su *et al.*, 1980; Homburger *et al.*, 1980; Shear *et al.*, 1976). Down regulation is defined as a decrease in the number of total cellular *beta* adrenergic receptors as detected with lipophilic radioligands such as [^{3}H]dihydroalprenolol or [^{125}I]IPIN, which bind to both surface and sequestered receptors on intact cells. The rate of down regulation of *beta* adrenergic receptors is

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ABBREVIATIONS: IPIN, iodopindolol; N_s, guanine nucleotide-binding protein for stimulation of adenylate cyclase activity; cyc$^-$, variant of S49 lymphoma cells which lacks N_s activity; UNC, variant of S49 lymphoma cells in which N_s is present but cannot interact with *beta* adrenergic receptors; H21a, variant of S49 lymphoma cells in which N_s is present but cannot interact with adenylate cyclase; WT, wild-type; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; B_{max}, number of binding sites per cell; N_i, guanine nucleotide-binding protein for inhibition of adenylate cyclase activity.

tissue-dependent ($T_{1/2}$ of 1–6 hr), is complete within 12 to 24 hr and is only reversible slowly (Su *et al.*, 1980; Homburger *et al.*, 1980; Doss *et al.*, 1981; Pittman *et al.*, 1984). In most cases, recovery from down regulation requires protein synthesis (Frederich *et al.*, 1983; Doss *et al.*, 1981). The relationship between sequestration and down regulation of *beta* adrenergic receptors is not well understood, and one purpose of the present study was to determine if sequestration is an obligatory step in the down regulation of *beta* adrenergic receptors.

Activation of adenylate cyclase by *beta* adrenergic receptors requires interaction of the *beta* adrenergic receptor with the stimulatory guanine nucleotide binding regulatory protein, N_s (Northup *et al.*, 1980). An important issue with regard to the molecular mechanisms involved in agonist-induced sequestration and down regulation of *beta* adrenergic receptors is the role of an interaction of *beta* adrenergic receptors with N_s in these processes. A useful approach for investigating this issue involves the use of mutants of S49 mouse lymphoma cells with specific lesions in various components of the *beta* adrenergic receptor-N_s-adenylate cyclase system. Cyc^- cells have functional *beta* adrenergic receptors and express adenylate cyclase activity, but lack functional N_s (Howlett *et al.*, 1979). UNC and H21a cells have functional *beta* adrenergic receptors, N_s and adenylate cyclase. However, in UNC cells interactions between *beta* adrenergic receptors and N_s do not occur (Sternweis and Gilman, 1979), whereas in H21a cells, interactions of N_s with adenylate cyclase do not occur (Salomon and Bourne, 1981).

Agonist-induced sequestration of *beta* adrenergic receptors occurs normally in UNC and cyc^- S49 cells, suggesting that interaction of *beta* adrenergic receptors with N_s is not involved in sequestration (Reynolds *et al.*, 1985; Mahan *et al.*, 1985). Agonist-induced down regulation of *beta* adrenergic receptors occurs in cyc^- and UNC S49 cells, but to a lesser extent than in WT cells (Mahan *et al.*, 1985). This suggests that 1) partial down regulation can occur in the absence of an interaction of *beta* adrenergic receptors with N_s and 2) sequestration of receptors is not a sufficient signal for maximal down regulation to occur.

Furthermore, it has been observed that there are several ligands (*e.g.*, pindolol and celiprolol) that do not stimulate adenylate cyclase but which do cause partial down regulation of *beta* adrenergic receptors in several types of cells (Neve *et al.*, 1985). Inasmuch as pindolol and celiprolol do not appear to promote the interaction of *beta* adrenergic receptors with N_s, these findings suggested that pindolol and celiprolol may activate selectively a mechanism of down regulation that does not involve interaction of *beta* adrenergic receptors with N_s. This hypothesis was tested by investigating the ability of pindolol and celiprolol to cause down regulation of *beta* adrenergic receptors in cyc^- and UNC mutants of S49 lymphoma cells.

Because agonist-induced sequestration of *beta* adrenergic receptors occurs normally in the absence of an interaction of *beta* adrenergic receptors with functional N_s, it is possible for a ligand (*e.g.*, pindolol or celiprolol) to induce sequestration without promoting the interaction of *beta* adrenergic receptors with N_s. It was therefore of interest to determine whether pindolol and celiprolol cause sequestration of *beta* adrenergic receptors in S49 cells.

Methods

Cell culture. Stock cultures of S49 lymphoma cells were maintained in spinner culture at a density of 0.1 to 1.0 × 10^6 cells/ml in Dulbecco's modified Eagle's medium supplemented with 10% horse serum (HyClone) in a humidified incubator containing 10% CO_2-90% air at 37°C.

Incubation protocol and binding assay for measurement of sequestration. Cells in growth medium (10^6 cells/ml) were centrifuged at 600 × *g* for 5 min at room temperature, resuspended in Leibowitz's L15 medium supplemented with 20 mM Na-HEPES (pH 7.4), 2% horse serum and 1 g/l of glucose (L15/HHG) and centrifuged again. Cells were resuspended in L15/HHG at a density of 2 × 10^6 cells/ml (50 ml total) and preincubated at 37°C for 30 min before the addition of drugs. Drugs were then added and the cells incubated for another 30 min at 37°C. This incubation was terminated by diluting the cell suspension into 200 ml of ice-cold L15/HHG and incubating the cells on ice for 10 min, after which the suspension was centrifuged at 600 × *g* for 5 min at 4°C. The cells were washed an additional 3 times with ice-cold L15/HHG and finally resuspended in ice-cold L15/HHG supplemented with additional horse serum (10% final concentration) at a density of 5 × 10^6 cells/ml.

To measure surface *beta* adrenergic receptors, aliquots (2.0 ml) of the above suspension (10^7 cells) were incubated in 35-mm culture dishes (Falcon) with 50 μl of L15/HHG containing various concentrations of [^{3}H]CGP-12177 (0.05–2.00 nM final concentration) and 50 μl of L15/HHG containing either no drug (total binding) or *dl*-propranolol (2 μM final concentration; nonspecific binding) for 18 hr at 6°C. The reaction was terminated by transferring the suspension to Schleicher and Schuell glass-fiber filters (No. 30) in a Millipore filtration apparatus and washing the filter-trapped cells with 20 ml of cold wash buffer (10 ml Tris, 154 mM NaCl, pH 7.5), after which the filter was transferred to a vial containing 10 ml of Budget Solve and radioactivity was determined by scintillation spectroscopy at an efficiency of 35%. The density (B_{max}) of binding sites for [^{3}H]CGP-12177 was calculated by Scatchard analysis (Scatchard, 1949), and these data were used as an estimate of the number of surface *beta* adrenergic receptors remaining after the 30-min incubation with drug.

Incubation protocol and binding assay for measurement of down regulation. To induce down regulation, cells grown in suspension culture were centrifuged at 600 × *g* for 5 min, resuspended in fresh growth medium at a density of 0.15 × 10^6 cells/ml, seeded in 150-mm culture dishes (Lux) and preincubated for 24 hr at 37°C in 10% CO_2-90% air. Drugs were added at various times over the next 24 hr. Isoproterenol was added at 0, 6, 12 and 18 hr, whereas celiprolol, pindolol and propranolol were added only at 0 and 12 hr. Cells were harvested 24 hr after the first drug addition (48 hr after seeding). To determine the time course of the observed effects, drugs were added 0 to 24 hr before the cells were to be harvested. Cells were harvested by centrifugation at 600 × *g* for 5 min at 4°C. The cell pellet was washed twice with 15 ml of ice-cold L15/HHG, after which the cells were resuspended in 15 ml of L15/HHG and incubated at 37°C for 15 min to allow bound drug to dissociate and for reversal of sequestration. The suspension was then centrifuged and cells were resuspended in ice-cold L15/HHG at a density of 2 × 10^6 cells/ml before being incubated with [^{125}I]IPIN.

To measure the number of total cellular *beta* adrenergic receptors, 0.15-ml aliquots of the above final suspension (0.3 × 10^6 cells) were incubated in polystyrene tubes (Sarstedt 55-463) with 50 μl of L15/HHG containing various concentrations of [^{125}I]IPIN (5–280 pM final concentration) and 50 μl of L15/HHG containing either no drug (total binding) or *dl*-propranolol (1 μM final concentration; nonspecific binding) for 30 min at 37°C. The reaction was stopped by the addition of 10 ml of wash buffer at room temperature, followed by filtration over Schleicher and Schuell glass-fiber filters (No. 30) on a Millipore filtration apparatus. The filters were washed with an additional 10 ml of wash buffer at room temperature, dried and counted in an LKB gamma counter. The density (B_{max}) of binding sites for [^{125}I]IPIN was determined by Scatchard analysis and used as an estimate of the number of total cellular *beta* adrenergic receptors (surface plus sequestered) on the cells.

Preparation of membranes and assay of adenylate cyclase activity. Cells from suspension culture (10^6 cells ml) were centrifuged

at 600 × g for 5 min at 4°C and washed twice with ice-cold Dulbecco's phosphate-buffered saline (Grand Island Biological Co., Grand Island, NY). The cells were resuspended in lysis buffer containing 5 mM Na-HEPES (pH 7.5), 1 mM $MgSO_4$ and 1 mM EDTA at a density of 10^7 cells/ml, allowed to swell for 10 min and then homogenized by hand with 20 strokes of a Dounce glass homogenizer. The suspension was centrifuged for 5 min at 900 × g at 4°C to pellet intact cells and nuclei. The supernatant containing plasma membrane fragments was then centrifuged at 35,000 × g for 20 min at 4°C, and the pellet was resuspended in 5 mM Na-HEPES (pH 7.5) at a protein concentration of 0.5 mg/ml.

Adenylate cyclase activity of this membrane suspension was determined by a modification (Minneman *et al.*, 1979) of the method of Salomon *et al.* (1974). [α^{32}P]ATP was purchased from New England Nuclear (Boston, MA). The assay solution consisted of 50 mM Na-HEPES (pH 7.5), 5 mM cyclic AMP, 0.25 mM ATP, 0.5 mM EGTA, 1 mM $MgCl_2$, 1 mM 1-methyl-3-isobutylxanthine, 100 μg/ml of creatine phosphokinase, 10 mM phosphocreatine, 30 μM GTP, 1 to 2 × 10^6 cpm per tube of [α-^{32}P]ATP, 50 μg of protein and drug in a final volume of 200 μl. Adenylate cyclase was assayed with no added drug (basal) or drug added for 10 min at 30°C. All experimental points were assayed in quadruplicate. Protein was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Results

The time course of the loss of surface *beta* adrenergic receptors (sequestration) and total cellular *beta* adrenergic receptors (down regulation) on WT S49 cells was determined during a 24-hr incubation with isoproterenol (1 μM; fig. 1). The number of surface *beta* adrenergic receptors was determined by Scatchard analysis of the binding of [^{3}H]CGP-12177 at 6°C for 18 hr on intact cells. Under these conditions, [^{3}H]CGP-12177 has been shown to label selectively surface *beta* adrenergic receptors on S49 cells (Mahan *et al.*, 1985), as well as C6 glioma cells (Hertel *et al.*, 1983b; Staehelin and Hertel, 1983) and astrocytoma cells (Toews *et al.*, 1985). The number of total cellular *beta* adrenergic receptors was determined by Scatchard analysis of the binding of [^{125}I]IPIN at 37°C for 30 min on the same preparation of treated cells. Incubation with 1 μM isoproterenol for 15 min caused a marked loss of surface *beta* adrenergic receptors without significantly affecting the number of total *beta* adrenergic receptors. Over the next 6 hr there was a substantial decrease in the number of total *beta* adrenergic receptors and a further reduction in the number of surface *beta* adrenergic receptors. After exposure to isoproterenol for 24 hr, the number of surface and total *beta* adrenergic receptors was decreased to 2 and 22%, respectively, of that in control cells.

Experiments in which the binding of [^{125}I]IPIN was inhibited by pindolol and celiprolol on intact WT S49 cells indicated that these drugs have K_i values of 1.1 nM (pindolol) and 2.0 μM (celiprolol) for *beta* adrenergic receptors on intact S49 cells (fig. 2). At concentrations 12 to 18 times the K_i values for these drugs, incubation of WT S49 cells with celiprolol or pindolol resulted in the loss of approximately half of the total cellular *beta* adrenergic receptors after a 24-hr incubation (fig. 3). The full agonist isoproterenol caused a 75% reduction in the number of receptors over the same incubation period. The decrease in the number of receptors observed when cells were incubated with pindolol or celiprolol was blocked by propranolol (fig. 3).

In membranes prepared from WT S49 cells, isoproterenol caused a 10-fold increase in adenylate cyclase activity over basal levels determined in the absence of drug (table 1). Pindolol and celiprolol did not increase adenylate cyclase activity

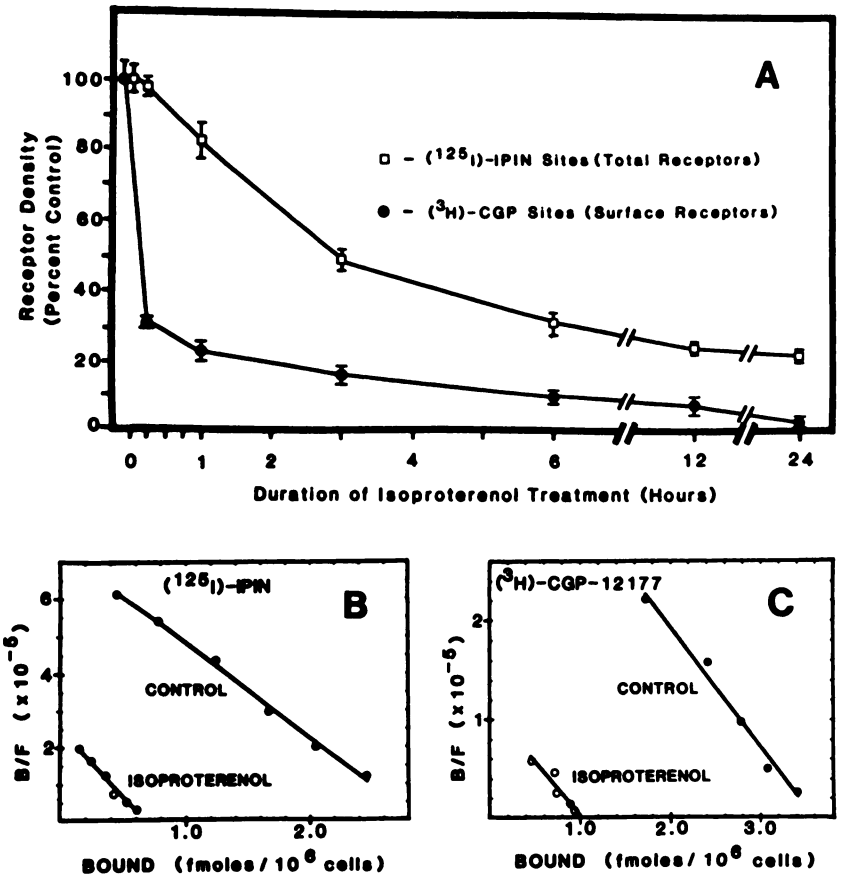

Fig. 1. Time course of isoproterenol-induced loss of total and surface *beta* adrenergic receptors on intact WT S49 cells. WT S49 cells in growth medium were exposed to 1 μM (−)-isoproterenol at 37°C for the time indicated, washed extensively, then divided into two groups to determine the number of total ([^{125}I]IPIN sites) and surface ([^{3}H]CGP sites) receptors as described under "Methods." A, time course of the loss of specific [^{125}I]IPIN and [^{3}H]CGP sites. The B_{max} in control cells was 1710 ± 43 ([^{125}I]IPIN) and 1713 ± 94 ([^{3}H]CGP) sites. The data are expressed as the percentage of control ± S.E.M. (n = 3) of sites remaining after the indicated time of exposure to 1 μM (−)-isoproterenol. B, representative Scatchard plots of specific [^{125}I]IPIN binding in intact control cells and cells treated with 1 μM isoproterenol for 24 hr. C, representative Scatchard plots of specific [^{3}H]CGP binding in intact control cells and cells treated with 1 μM isoproterenol for 15 min. B/F, bound/free.

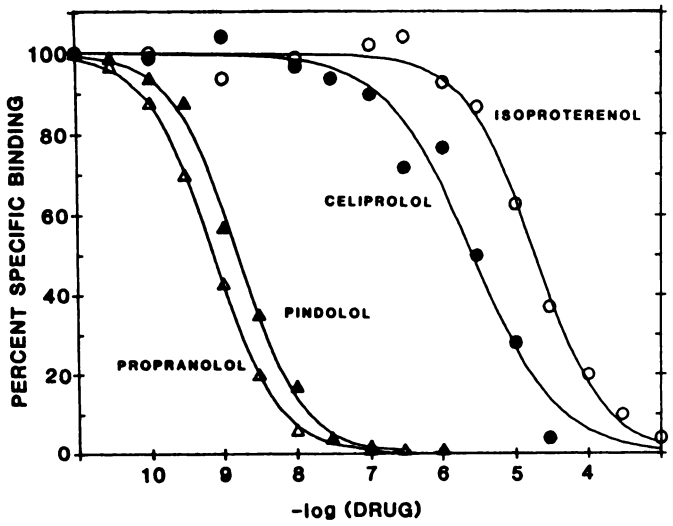

Fig. 2. Inhibition of the binding of [^{125}I]IPIN to *beta* adrenergic receptors on intact WT S49 cells by isoproterenol, celiprolol, pindolol and propranolol. WT S49 cells were harvested, washed and resuspended in L15/HHG. Aliquots of cells (3–4 × 10^5/0.25 ml) were incubated for 30 min at 37°C with [^{125}I]IPIN (54 pM) and various concentrations of drugs. The reaction was stopped by rapid filtration and a single wash as described under "Methods." Nonspecific binding was determined in the presence of 1 μM *dl*-propranolol. The data are expressed as the percentage of specific binding of [^{125}I]IPIN *vs.* the concentration of drug. K_i values, determined by the method of Cheng and Prusoff (1973), were 6.1 μM (○, isoproterenol), 2.0 μM (●, celiprolol), 1.1 nM (▲, pindolol) and 0.2 nM (△, propranolol).

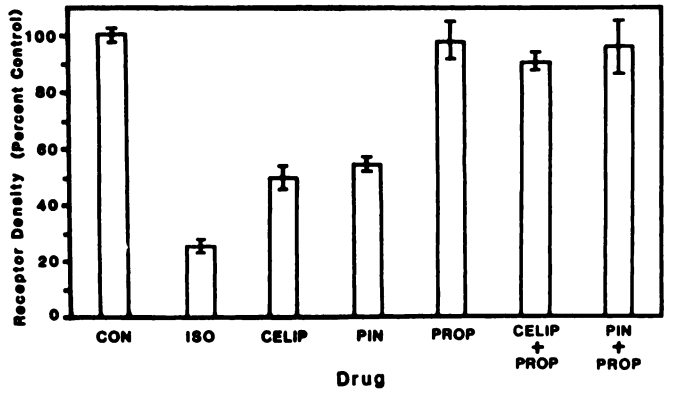

Fig. 3. Density of *beta* adrenergic receptors in WT S49 cells after treatment with agonists or antagonists for 24 hr. WT S49 cells were exposed to the indicated drugs for 24 hr at 37°C, washed and incubated with [^{125}I]-IPIN to determine the number of total cellular *beta* adrenergic receptors as described under "Methods." The drug concentrations were (−)-isoproterenol, 2.5 μM; *dl*-celiprolol, 25 μM; *dl*-pindolol, 20 nM; and (−)-propranolol, 200 nM. Data are expressed as the percentage ± S.E.M. (n = 3) of the density of *beta* adrenergic receptors on control cells. The density of receptors in control cells in the absence of drug was 1874 ± 33 receptors per cell. CON, control; ISO, isoproterenol; CELIP, celiprolol; PIN, pindolol; PROP, propranolol.

over basal levels at concentrations 25 to 45 times their K_i value (table 1).

To determine whether pindolol and celiprolol could induce sequestration of *beta* adrenergic receptors, WT S49 cells were incubated for 30 min at 37°C with pindolol, celiprolol or isoproterenol, after which the cells were washed extensively at 4°C. Isoproterenol (2 μM) caused a 65% loss of surface *beta* adrenergic receptors (table 2). Under the same conditions, pindolol and celiprolol did not cause any loss of surface *beta*

TABLE 1

Efficacy of atypical agonists in membranes prepared from WT S49 cells

Membranes prepared from intact WT S49 cells were prepared and assayed for adenylate cyclase activity as described under "Methods." The values for adenylate cyclase activity are expressed in picomoles of cyclic AMP formed per minute per milligram of protein and are the mean ± S.E.M. of three experiments. The concentrations used were 25 times (celiprolol), 45 times (pindolol) and 0.16 times (isoproterenol) the K_i value of the drug determined in experiments carried out with WT S49 cells (fig. 1). Fold stimulation represents adenylate cyclase activity in the presence of drug divided by basal adenylate cyclase activity.

Drug	Adenylate Cyclase Activity	Fold Stimulation
Basal (0 μM)	3.9 ± 0.4	1.0
(−)-Isoproterenol (1 μM)	39.5 ± 7.9	10.1
Celiprolol (50 μM)	4.2 ± 0.4	1.1
Pindolol (0.05 μM)	3.8 ± 0.5	1.0

TABLE 2

Loss of surface *beta*-adrenergic receptors on intact WT S49 cells after a 30-min incubation with isoproterenol, celiprolol and pindolol

WT S49 cells (2 × 10^6/ml) were incubated for 30 min at 37°C with the indicated drug and then with [^{3}H]CGP-12177, to determine the number of surface *beta* adrenergic receptors (B_{max}) as described under "Methods." Values shown are means ± S.E.M. (n = 3) of the percentage of loss of surface *beta* receptors compared to control cells incubated with no drugs for 30 min. The K_D and B_{max} values of control cells were 69 pM and 2014 receptors per cell, respectively. The K_D values in treated cells were 80 pM (isoproterenol), 124 pM (celiprolol) and 189 pM (pindolol).

Drug	% Loss of Surface *Beta* Receptors
(−)-Isoproterenol (2 μM)	65.0 ± 0.7
Celiprolol (50 μM)	−1.9 ± 4.8
Pindolol (0.05 μM)	−2.3 ± 3.9

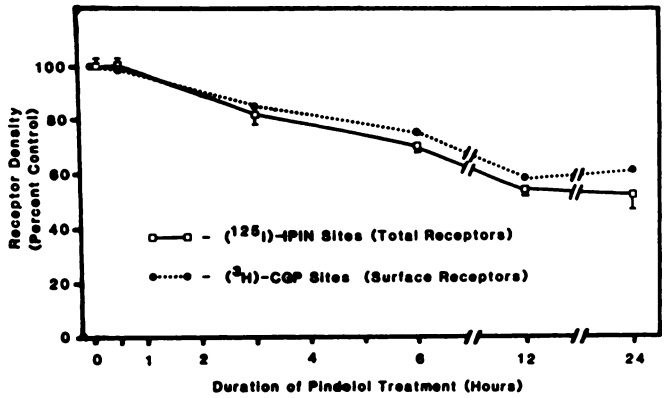

Fig. 4. Time course of pindolol-induced loss of total and surface *beta* adrenergic receptors on intact WT S49 cells. WT S49 cells were exposed to 50 nM pindolol for the indicated times at 37°C, washed extensively, then divided into two groups to determine the number of total ([^{125}I]IPIN sites) and surface ([^{3}H]CGP sites) receptors as described under "Methods." The B_{max} (computed from Scatchard analysis of saturation curves) in control cells was 1423 ([^{3}H]CGP-12177; n = 1) and 1515 ± 26 ([^{125}I]IPIN; n = 3). The data are expressed as the percentage of control (no drug) of binding sites per cell remaining after exposure to 50 nM pindolol for the indicated times.

adrenergic receptors at concentrations greater than 25 times their K_i values.

The temporal relationship between the loss of surface and total *beta* adrenergic receptors induced by pindolol on WT S49 cells was determined over a 24-hr incubation period (fig. 4). There was no loss of surface or total *beta* adrenergic receptors after exposure to pindolol for 30 min. Thereafter, there was a parallel loss of surface and total *beta* adrenergic receptors that reached completion after 12 hr.

To determine whether interaction of *beta* adrenergic receptors with N_s is required for pindolol and celiprolol to cause

down regulation of receptors, the extent of down regulation caused by these drugs was determined in two mutant lines of S49 cells (UNC and cyc$^-$) in which a functional interaction of *beta* adrenergic receptors with N_s does not occur. After a 24-hr incubation, isoproterenol caused a 73% decrease in the number of total cellular *beta* adrenergic receptors in WT S49 cells, a 90% decrease in H21a cells, but only a 42 and 58% decrease in cyc$^-$ and UNC cells, respectively (table 3). However, although incubation of cells with pindolol and celiprolol resulted in a decrease in total cellular *beta* adrenergic receptors in WT (45 and 50% decrease, respectively) and H21a S49 cells (37 and 42% decrease, respectively), they did not cause a decrease in total cellular *beta* adrenergic receptors in either cyc$^-$ or UNC cells at concentrations greater than 25 times their K_i values. Propranolol, a typical *beta* adrenergic antagonist, did not significantly alter the number of total cellular *beta* adrenergic receptors in either WT or cyc$^-$ S49 cells after a 24-hr incubation. None of these drugs (isoproterenol, propranolol, pindolol or celiprolol) had any significant effect on the growth rate of WT S49 cells in a 24-hr incubation (data not shown).

Discussion

Recent studies have led to important observations with respect to the mechanisms involved in agonist-induced down regulation of *beta* adrenergic receptors. Mahan *et al.* (1985) have reported that in mutant S49 lymphoma cells in which the interaction of *beta* adrenergic receptors with N_s does not occur (UNC and cyc$^-$), isoproterenol causes normal sequestration of the receptor but only partial down regulation. These authors concluded that sequestration of *beta* adrenergic receptors is not a sufficient signal to induce maximal down regulation, and that significant down regulation can occur in the absence of an interaction of *beta* adrenergic receptors and N_s.

Neve *et al.* (1985) reported that two atypical agonists at *beta* adrenergic receptors, pindolol and celiprolol, which do not stimulate adenylate cyclase, are able to cause partial (half-maximal) down regulation of *beta* adrenergic receptors in C6 glioma cells. Because pindolol and celiprolol did not appear to promote the interaction of *beta* adrenergic receptors with N_s, these authors concluded that *beta* adrenergic receptors can be down regulated without prior interaction with N_s. The results of these studies suggest that there may be several mechanisms of down regulation of *beta* adrenergic receptors, one of which does not require the interaction of the receptors with N_s.

The present experiments were carried out to test the hypothesis that there are two mechanisms of agonist-induced down regulation of *beta* adrenergic receptors in WT S49 cells, one of which involves the sequestration of *beta* adrenergic receptors but does not involve interaction of *beta* adrenergic receptors with N_s, and another that requires the interaction of *beta* adrenergic receptors with N_s but does not involve sequestration of *beta* adrenergic receptors. The observation that pindolol and celiprolol cause partial down regulation in WT S49 cells suggested that these drugs may activate selectively only one of these mechanisms. The goal of the present study was to explore this hypothesis by determining whether pindolol and celiprolol induce sequestration in WT S49 cells, and by investigating the ability of these drugs to cause down regulation in S49 mutants in which the functional interaction of *beta* adrenergic receptors with N_s does not occur.

Celiprolol and pindolol induced down regulation of *beta* adrenergic receptors on WT S49 cells, although the extent of this effect was smaller than that of the full agonist isoproterenol. Consistent with their effects in other cell types, pindolol and celiprolol did not stimulate adenylate cyclase on WT S49 cells. Thus, the mechanism by which these drugs cause down regulation of *beta* adrenergic receptors does not involve activation of adenylate cyclase or the generation of cyclic AMP. At concentrations greater than 25 times their K_i, pindolol and celiprolol did not cause sequestration of *beta* adrenergic receptors after an incubation for 30 min. This suggests that the mechanism by which they cause down regulation does not involve sequestration of *beta* adrenergic receptors, and that down regulation of *beta* adrenergic receptors can occur in the absence of sequestration. The observation that pindolol causes the loss of total cellular *beta* adrenergic receptors at the same rate as the loss of surface *beta* adrenergic receptors raises the possibility that pindolol may induce the degradation of *beta* adrenergic receptors in or near the plasma membrane and not in an intracellular compartment. Such a mechanism is different from that described for the down regulation of a number of peptide receptors including insulin and EGF (Beguinot *et al.*,1984; Knutson *et al.*, 1983). In these systems, agonists induce the internalization of receptors into a vesicular compartment called endosomes or receptosomes. The receptors are then either recycled back to the plasma membrane (upon removal of the agonist) or are transported to another intracellular compartment in which they are degraded. This latter process occurs in the continued presence of agonist. In the case of these peptide receptors, the process of internalization appears to be an obligatory step in agonist-induced down regulation of these receptors, and the receptors are degraded within an intracellular compartment. Much less is known about the mechanisms involved in the sequestration and down regulation of *beta* adrenergic receptors, but the observation that down regulation can occur in the absence of sequestration suggests that these regu-

TABLE 3

Loss of total *beta* adrenergic receptors in mutant S49 cells after treatment with typical and atypical agonists for 24 hr

S49 cells were exposed to the indicated drugs for 24 hr at 37°C, washed and incubated with [^{125}I]IPIN to determine the number of total cellular *beta* adrenergic receptors as described under "Methods." Data are expressed as the percentage ± S.E.M. (n = 3) of the density of *beta* adrenergic receptors on control cells after treatment for 24 hr in the absence of drug. The density of *beta* adrenergic receptors (receptors per cell) on control cells was: 1874 ± 33 (WT), 2827 ± 117 (H21a), 3607 ± 54 (UNC) and 2898 ± 60 (cyc$^-$).

Drug	Receptor Density, % Control			
	WT	H21a	Cyc$^-$	UNC
Control	100.0 ± 2.2	100.0 ± 4.1	100.0 ± 2.1	100.0 ± 1.5
(−)-Isoproterenol (1 μM)	26.7 ± 2.1	10.3 ± 0.4	58.4 ± 1.3	41.9 ± 2.9
Celiprolol (50 μM)	50.0 ± 4.4	58.0 ± 3.2	102.3 ± 3.6	104.1 ± 3.2
Pindolol (0.05 μM)	55.3 ± 2.3	63.4 ± 0.7	107.8 ± 0.3	99.6 ± 7.7
(−)-Propranolol (0.33 μM)	98.3 ± 6.2		99.1 ± 0.3	

latory mechanisms may be fundamentally different for *beta* adrenergic receptors than for receptors for peptides.

Based on the observations that the binding of celiprolol and pindolol to *beta* adrenergic receptors on C6 glioma membranes is not affected by GTP and does not stimulate adenylate cyclase activity, Neve *et al.* (1985) concluded that these drugs do not promote the functional interaction of *beta* adrenergic receptors with N_s. However, the observation that pindolol and celiprolol cause down regulation in S49 cells in which normal interaction of *beta* adrenergic receptors with N_s occurs (WT and H21a), but not in S49 cells in which the interaction of *beta* adrenergic receptors with N_s cannot occur (cyc^- and UNC), suggests that pindolol and celiprolol promote an atypical interaction of *beta* adrenergic receptors with N_s which is productive with respect to down regulation but not productive with respect to stimulation of adenylate cyclase. An alternative explanation is that pindolol and celiprolol promote a typical, but very inefficient, interaction of *beta* adrenergic receptors with N_s. A small increase in cyclic AMP levels might be undetectable using conventional assays but could still lead to a slow down regulation of *beta* adrenergic receptors. Pittman *et al.* (1984) demonstrated that weak partial agonists (with respect to stimulation of adenylate cyclase) could cause down regulation of *beta* adrenergic receptors to the same extent, but at a slower rate, as full agonists in a 24-hr incubation. However, results observed in studies with pindolol and celiprolol are different fundamentally from those obtained with the partial agonists used previously, because although the maximal effect of pindolol or celiprolol is reached within 24 hr, the extent of down regulation is only about half that of the full agonist isoproterenol. We thus favor the interpretation that the interaction of *beta* adrenergic receptors with N_s can lead to two discrete and separable effects, the stimulation of adenylate cyclase and the induction of down regulation of *beta* adrenergic receptors, and that a particular ligand can cause one of these effects without the other. In S49 cells, the interaction of *beta* adrenergic receptors with N_s also leads to the inhibition of Mg^{++} influx as well as the stimulation of adenylate cyclase activity (Maguire and Erdos, 1980; Erdos and Maguire, 1980). These two events are regulated independently, suggesting that they are also discrete and separable consequences of the interaction of *beta* adrenergic receptors with N_s. The possible relationship between Mg^{++} influx and down regulation is not known at present.

It is also possible that down regulation involves the interaction of the *beta* adrenergic receptor with only one of the subunits of N_s, rather than with the functional oligomeric form of N_s. Functional N_s is a heterotrimer consisting of *alpha*-, *beta*- and *gamma*-subunits (Hildebrandt *et al.*, 1984; Bokoch *et al.*, 1984). Cyc^- cells are devoid of the stimulatory *alpha*-subunit (Harris *et al.*, 1985), although the *beta*-subunit is present in approximately normal amounts (Northup *et al.*, 1983). Thus, because the *beta*-subunit of N_s/N_i is present in cyc^- cells, one cannot rule out the possibility that the interaction of the *beta*-adrenergic receptor with the *beta*-subunit of N_s, rather than with the heterotrimer of *alpha-beta-gamma* of N_s, is involved in down regulation and/or sequestration in cyc^- cells.

Thus, pindolol and celiprolol appear to cause the down regulation of *beta* adrenergic receptors by a mechanism which does not involve the sequestration of *beta* adrenergic receptors but does require the interaction of *beta* adrenergic receptors with N_s. This raises the question of the mechanism of isoproterenol-induced down regulation which occurs in cyc^- and UNC cells. Because down regulation in these cells does not involve an interaction of *beta* adrenergic receptors with N_s, this process appears to be fundamentally different from that stimulated by pindolol and celiprolol in WT cells. Inasmuch as sequestration occurs normally in cyc^- and UNC cells, it is reasonable to speculate that down regulation in these cells involves the sequestration of *beta* adrenergic receptors. This hypothesis is consistent with the observation that the extent of down regulation in cyc^- cells is less than that observed in WT cells and may represent one of two mechanisms involved in this process in WT S49 cells.

A model consistent with the above observations can be summarized as follows: there are two mechanisms of down regulation of *beta* adrenergic receptors in WT S49 cells, one of which involves the interaction of *beta* adrenergic receptors with N_s and one of which does not. The mechanism that involves the interaction of *beta* adrenergic receptors with N_s occurs in or near the plasma membrane, does not involve sequestration of receptors and does not occur in cyc^- or UNC cells. The other mechanism involves the interaction of *beta* adrenergic receptors with a cellular component other than N_s, requires the sequestration of *beta* adrenergic receptors and occurs in mutant S49 lymphoma cells. According to this model, pindolol and celiprolol activate selectively the mechanism that requires interaction of *beta* adrenergic receptors with N_s but does not involve sequestration of *beta* adrenergic receptors.

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