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Genetic analysis of β-adrenergic receptor internalization and down-regulation

(S49 lymphoma cell/receptor desensitization/cyclic AMP/receptor turnover/guanine nucleotide binding protein)

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ABSTRACT **We have used wild-type and variants of the T-lymphoma cell line S49 to explore internalization and down-regulation of adenylate cyclase-linked β-adrenergic receptors. Internalization was defined by the loss in "surface receptors" detected at 4°C on intact cells by the antagonists [^{3}H]CGP-12177 or [^{125}I]iodocyanopindolol, whereas down-regulation was defined as the loss in total cellular content of receptors ([^{125}I]iodocyanopindolol binding assayed at 37°C). In wild-type cells, the β-adrenergic agonist isoproterenol induced a rapid ($t_{1/2}$, ≈1 min) and reversible loss in surface receptors. The surface sites were lost at a rate similar to the rate of desensitization of β-adrenergic receptor-mediated cyclic AMP generation of S49 cells. A series of S49 variants (cyc$^-$, UNC, H21a) having lesions in N_s (the guanine nucleotide binding protein that couples β-receptors to adenylate cyclase) or with absent cAMP-dependent protein kinase activity (kin$^-$), had a loss in surface sites that was equivalent to that of wild-type cells. By contrast, S49 variant cells having lesions in N_s showed variable rates and extents of down-regulation of β-adrenergic receptors. In wild-type and kin$^-$ S49 cells, β-receptors down-regulated with a $t_{1/2}$ of ≈4 hr. Down-regulation was blunted in the cyc$^-$ and UNC variants that have altered coupling of receptors to N_s, but it was faster in the H21a variant that retains receptor–N_s interaction. Recovery of receptors after down-regulation occurred at a similar rate ($t_{1/2}$, ≈6 hr) in wild-type, UNC, and H21a cells. These results demonstrate that internalization of β-adrenergic receptors may be necessary, but is not sufficient, to explain agonist-induced receptor down-regulation in S49 cells. The variable expression in the development of down-regulation in S49 variants implies that receptor–N_s interaction regulates the fate of receptors linked to the stimulation of adenylate cyclase.**

Agonist-promoted desensitization appears to be a general homeostatic mechanism by which target cells modulate responsiveness to agents acting at cell-surface receptors (1, 2). For many hormonal systems, this desensitization (also termed refractoriness, tolerance, or tachyphylaxis) is closely linked to an agonist mediated decrease (down-regulation) of receptors (1–3). For other receptor systems, in particular many of those linked to adenylate cyclase, such as the β-adrenergic receptor, agonist-mediated down-regulation occurs more slowly than the loss in target cell response (4, 5). This earlier loss in response correlates with a decrease in receptor affinity for agonists, an event that has recently been proposed to represent an internalization of cell-surface receptors to distinct intracellular sites (6–11). Thus, a current model of desensitization is that receptor internalization precedes and initiates receptor down-regulation. We have used a genetic approach involving a unique model system (10, 12) to explore this issue: murine S49 lymphoma cells and S49 clonal variants with lesions in cAMP-dependent protein kinase or N_s (the guanine nucleotide binding protein that couples receptor occupancy to activation of adenylate cyclase). We show here that internalization of β-adrenergic receptors in S49 cells is independent of both N_s and cAMP-dependent protein kinase, but that N_s influences both the rate and extent of down-regulation of receptors.

METHODS

Cell Lines and Cell Growth. The S49 variants used for the experiments described here include UNC [cells that have an electrophoretically altered N_s and defective interaction of N_s with hormone receptors (R–N_s interaction) (13, 14)], H21a [cells whose N_s is defective in interaction with catalytic (C) component of adenylate cyclase (N_s–C interaction) (15)], cyc$^-$ [cells that lack the 45,000 Da α-subunit of N_s (16), and kin$^-$ [cells with absent cAMP-dependent protein kinase activity (17)]. Although detailed genetic analyses have not been provided for all these variants, UNC, H21a, and cyc$^-$ fail to complement one another in somatic cell hybrids and in subcellular reconstitution assays and, thus, are all presumed to have a lesion in the α-subunit of N_s (18); none of these variants generates cAMP in response to hormonal agonists (β-adrenergic, prostaglandin E_1). kin$^-$ cells, which have transdominant regulatory mutations in the expression of the catalytic subunit of cAMP-dependent protein kinase activity, show no cAMP-mediated responses (17, 19, 20). Cells were grown in liquid suspension cultures in Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% horse serum. Cells were used only during logarithmic phase growth.

Agonist Incubation Protocols. In experiments examining agonist-induced loss and return of surface sites, cells were washed free of growth medium and resuspended in DME medium/20 mM Hepes, pH 7.4/1 mg of bovine serum albumin per ml, at 37°C at a final concentration of ≈3 × 10^6 cells per ml. Cells were then incubated at 37°C with 1 μM (−)-isoproterenol, as described (10); superoxide dismutase and catalase (each at 10 μg/ml) were included to prevent catecholamine oxidation (35). Incubations were terminated by dilution of cells with ice-cold DME medium/20 mM Hepes, pH 7.4/bovine serum albumin (1 mg/ml)/10% horse serum, and washed 3 times by centrifugation (300 × *g* × 5 min, 4°C). Binding studies on washed cells were performed as described below. In experiments examining the development and reversal of down-regulation, cells were incubated in growth medium (DME medium/10% horse serum) at 37°C for various times in the presence of 1 μM (−)-isoproterenol and superoxide dismutase and catalase, each at 10 μg/ml. After this incubation, cells were washed 3 times by centrifugation and resuspended directly for binding assays or re-

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Abbreviations: N_s, guanine nucleotide binding protein that couples stimulatory receptors to adenylate cyclase; C, catalytic moiety of adenylate cyclase.

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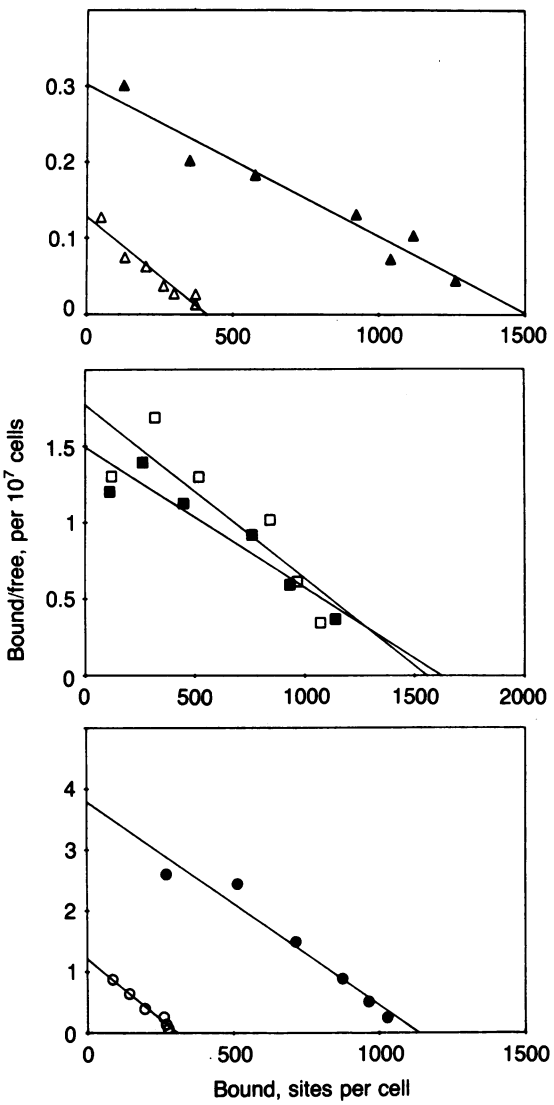

FIG. 1. Saturation binding isotherms of β-adrenergic receptors on intact wild-type S49 cells after 15 min and 18 hr of incubation with agonist. (*Upper*) [^{3}H]CGP saturation binding isotherm (shown as Scatchard analysis) for cells incubated with isoproterenol for 15 min. Wild-type S49 cells were incubated with (△) or without (▲) 1 μM (−)-isoproterenol for 15 min at 37°C, and washed cells were incubated in radioligand binding studies with various concentrations of [^{3}H]CGP (0.018–2.1 nM) and DME medium/Hepes/bovine serum albumin (total) or 1 μM (−)-propranolol isoproterenol (nonspecific) in a final vol of 0.5 ml for 18 hr at 4°C. Identical data were obtained in a duplicate experiment. Values for equilibrium dissociation constant (K_d) and maximal binding (B_{max}) in control and (−)-isoproterenol-treated S49 cells were 0.16×10^{-9} M and 1496 sites per cell, and 0.11×10^{-9} M and 410 sites per cell, respectively, in the experiment shown here. (*Middle*) [^{125}I]Iodocyanopindolol saturation binding isotherms for S49 cells incubated with agonist for 15 min. Wild-type S49 cells were treated with (□) or without (■) 1 μM (−)-isoproterenol and washed; binding of various concentrations of [^{125}I]iodocyanopindolol (3–105 pM) was assessed in an assay conducted for 1 hr at 37°C. Identical data were obtained in a duplicate experiment. K_d and B_{max} values obtained for [^{125}I]iodocyanopindolol binding in control and (−)-isoproterenol-treated cells were 36×10^{-12} M and 1642 sites per cell and 29×10^{-12} M and 1542 sites per cell, respectively. (*Lower*) [^{125}I]Iodocyanopindolol saturation binding isotherms for S49 cells incubated with agonist for 18 hr. Wild-type S49 cells were incubated with (○) or without (●) 1 μM (−)-isoproterenol in growth medium (DME medium/horse serum) at 37°C in a humidified 90% air/10% CO_2 incubator for 18 hr and then washed; binding of iodocyanopindolol was assessed in an assay conducted for 1 hr at 37°C. Identical results were obtained in several separate experiments with 1 μM isoproterenol and with the β_2-adrenergic agonist terbutaline (1 μM). In the experiment shown, the K_d and B_{max} values for control and isoproterenol-incubated cells were 10×10^{-12} M and 1130 sites per cell and 9×10^{-12} M and 310 sites per cell, respectively.

turned to growth conditions and binding assays conducted at later times as described below.

Radioligand Binding Studies. β-adrenergic receptors were assessed in intact S49 cells using the radioligands [^{3}H]CGP-12177 (CGP) and [^{125}I]iodocyanopindolol. Both radioligands are β-adrenergic antagonists and binding of both compounds to intact S49 cells demonstrates properties (kinetics, saturability, stereoselectivity, and rank order of potency of adrenergic agents) expected for β_2-adrenergic receptors (refs. 10 and 22; data not shown). Washed cells (≈4×10^6) were incubated in quadruplicate with CGP (0.018–2.1 nM in saturation isotherms) and DME medium/20 mM Hepes, pH 7.4/bovine serum albumin (1 mg/ml), containing horse serum at a final concentration of 10% in the absence (total) or presence (nonspecific) of 1 μM (−)-propranolol in a final vol of 0.5 ml for 18 hr at 4°C. This incubation period insures equilibrium of specific binding (total minus nonspecific) at all concentrations of CGP (data not shown). Incubation of intact cells under these conditions identifies cell-surface receptors (ref. 22; data not shown). Binding was terminated by dilution, filtration, and washing of filtered cells as described (10) except that buffer was used at 4°C. Samples were counted in a Beckman LS-7000 liquid scintillation counter with 46% counting efficiency.

Iodocyanopindolol binding was evaluated using two different protocols. In experiments assessing loss of surface sites, washed cells (8×10^5) were incubated with iodocyanopindolol (3–110 pM in saturation isotherms) in DME medium/20 mM Hepes, pH 7.4/bovine serum albumin (1 mg/ml), containing a final concentration of 12.5% horse serum in the absence or presence of 1 μM (−)-propranolol or 1 μM unlabeled CGP in a final vol of 0.5 ml for 18 hr at 4°C. Binding was terminated by dilution, filtration, and washing of filtered cells as described (10), except that the buffer used was phosphate-buffered saline at 37°C. Radioactivity on filters was counted in a γ-counter at 80% efficiency. In experiments assessing down-regulation, washed cells were incubated in a final vol of 0.5 ml for 1 hr at 37°C, and binding was terminated as described (10). Inclusion of horse serum in binding incubations at 4°C was necessary to maintain cell viability. For incubations with CGP at high cell density, 10% horse serum was sufficient; at lower cell density (by a factor of 5–10) in iodocyanopindolol experiments, 12.5% horse serum was required.

RESULTS

In these experiments, we examined agonist-mediated internalization and down-regulation of β-adrenergic receptors in S49 lymphoma cells. These events were assessed using the two antagonist radioligands, CGP and iodocyanopindolol. CGP, a hydrophilic compound, is membrane impermeant and binds selectively to cell surface receptors on S49 cells (refs. 22 and 23; data not shown). Iodocyanopindolol is permeable across the plasma membrane when binding studies are conducted at 37°C, and it identifies intracellular as well as cell-surface receptors. However, when iodocyanopindolol is incubated with cells at 4°C, as shown below, this radioligand, like CGP, can be used to assess cell-surface receptors. Thus, in the experiments to be described, binding of radioligands at 4°C provided information regarding cell-surface receptors (and internalization of these sites promoted by agonist) and binding at 37°C was used to assess loss of total cellular content of receptors (i.e., down-regulation).

Incubation of wild-type S49 cells with the β-agonist (−)-isoproterenol (1 μM) for 15 min at 37°C followed by exten-

sive washing and binding at 4°C resulted in a 60%–70% loss in CGP sites (Figs. 1 and 2). A similar loss in the binding of [^{125}I]iodocyanopindolol at 4°C was also observed (59% ± 5% loss; mean ± SEM; n = 4) to cells treated with 1 μM (−)-isoproterenol for 15 min. The remaining CGP (Fig. 1; ref. 25) and iodocyanopindolol (data not shown) sites had unaltered affinity for radioligand, indicating that the loss of sites after incubation with isoproterenol was not attributable to agonist retained during the washing procedure. Identical treatment of wild-type S49 cells followed by 37°C washing and binding of iodocyanopindolol at 37°C (Fig. 1, *Middle*) demonstrated that the loss in sites seen in studies conducted at 4°C did not represent an alteration in total cellular receptor number but rather a selective and reversible loss of surface receptors induced by isoproterenol (Fig. 2). Kinetic analysis indicated that cells incubated with 1 μM isoproterenol rapidly ($t_{1/2}$, ≈1 min) lost surface sites (Fig. 2, *Left*). This rapid loss of sites was similar to the rates of desensitization of cAMP generation and of a time-dependent decrease in agonist affinity of β-adrenergic receptors that we have observed on these cells (10). Upon removal of agonist (Fig. 2, *Right*), the sites returned to the surface at a rate ($t_{1/2}$, ≈16 min) slower than these sites were lost. The lines shown for loss and recovery of surface sites in Fig. 2 were each derived from fitting the data to rates described by single exponentials.

Incubation of each of the S49 variants with 1 μM (−)-isoproterenol for 15 min (Fig. 3) decreased CGP sites to an equivalent extent as in wild-type cells, thus showing that the α-subunit of N_s, activation of adenylate cyclase, cAMP itself, and cAMP-dependent protein phosphorylation are not required for the isoproterenol-mediated decrease in surface sites. A loss in CGP sites after incubation of cyc$^-$ S49 cells with isoproterenol was recently also observed by Hertel *et al.* (25).

In contrast with the rapid rate of loss of surface receptors induced by brief exposure to agonist, prolonged exposure of wild-type and variant S49 cells to agonist lead to quite different patterns of down-regulation. Loss of total cellular receptors could be detected (≈20%) in wild-type cells by 1 hr, but the steady state of down-regulation was not achieved until ≥12 hr (Fig. 1, *Lower*; Fig. 4, *Left*). Although wild-type and kin$^-$ S49 cells had relatively similar kinetics and extents of down-regulation, each of the variants having altered or absent N_s yielded a distinct pattern. H21a, the variant having an alteration in N_s–C interaction, down-regulated its receptors more rapidly than did wild-type cells. Both cyc$^-$ (absent of α-N_s) and to a lesser extent UNC cells (altered R–N_s interaction) showed a blunted isoproterenol-mediated loss in β-adrenergic receptors. These latter findings in intact cyc$^-$ cells are similar to previous reports on receptors in membranes prepared from agonist-incubated cyc$^-$ cells (15, 26–28).

The kinetics for reversal of down-regulation in intact S49 cells ($t_{1/2}$, ≈6 hr) was similar to the rate at which down-regulation occurred (Fig. 4) but was markedly slower than that for reversal of internalization (Fig. 2, *Right*). Although demonstrating different patterns for development of down-regulation, H21a and UNC cells had rates of receptor recovery from down-regulation that were similar to that of wild-type S49 cells. Therefore, lesions in R–N_s or N_s–C interaction appear not to influence either the extent or kinetics of receptor recovery from down-regulation.

DISCUSSION

Although much evidence points to the distinction between early and late events involved in cellular desensitization to catecholamines and other hormones (5, 7, 10, 11, 22, 27), the use of S49 variants to examine this problem provides several new insights. Most previous studies have emphasized properties of receptors in isolated membranes, whereas our attention has focussed on using intact cells. Our previously published work and that of others has demonstrated rapid decreases in agonist affinity, as detected in assays with several types of intact cultured cells (10, 11, 21, 29, 30). Given the hydrophilic nature of most agonists, rapid movement of receptors to a poorly accessible location is an event that may contribute to such decreases in agonist affinity as well as to the desensitization of adenylate cyclase, which occurs over a similar time course (10, 27, 31). Perhaps inaccessibility of

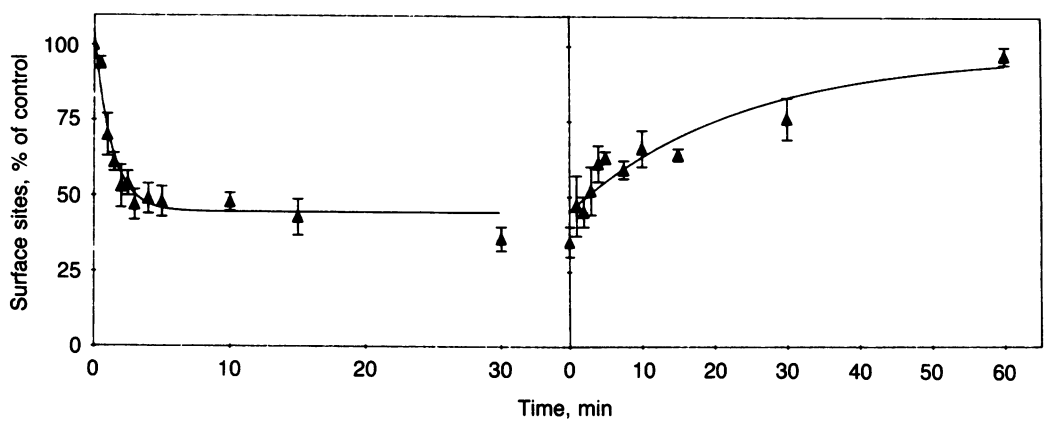

FIG. 2. Kinetics of loss and recovery of surface β-adrenergic receptors on wild-type S49 cells. (*Left*) Cells were incubated with 1 μM (−)-isoproterenol at 37°C for various lengths of time, then diluted and washed at 4°C. Binding of iodocyanopindolol was then conducted for 18 hr at 4°C. The decrease in surface sites was fit to the equation $R_t = R_L \exp{-k_L t} + R_s$, where R_t are receptors measured at time t; R_L is the magnitude of receptor loss (as % of control); k_L is the rate constant of receptor loss; and R_s is a constant for the steady-state level of retained receptors (as % of control). This fit generated the line shown and gave a value of k_L = 0.77 min^{-1} and, therefore, a $t_{1/2}$ of receptor loss of 0.9 min. Values for R_L and R_s were 60% and 45% of control, respectively. (*Right*) Cells were incubated with 1 μM (−)-isoproterenol for 15 min at 37°C, diluted and washed at 4°C, rewarmed at 37°C for various lengths of time, then diluted and washed. Binding of iodocyanopindolol was then conducted for 18 hr at 4°C. The recovery of surface sites was fit to the equation $R_t = R_s - R_O \exp{-k_a t}$, where R_t are the receptors measured at time t; R_s is the steady-state level of receptors after recovery (defined as 100%); R_O is the loss of receptors from the surface at the time of removal of agonist; and k_a is the rate constant for receptor reappearance. The fit generated the line shown and gave a value of k_a = 0.042 min^{-1} and, therefore, a $t_{1/2}$ of recovery of 16.8 min. The value obtained for R_O was 53%. Rate constants for loss and recovery of receptors were obtained by nonlinear least-squares regression analysis of experimental data according to Marquardt (36) on a Tektronix 4051 computer. Data are shown as surface sites relative to control cells and represent mean values for 4 experiments during loss of surface sites and 2–3 experiments for recovery of surface sites.

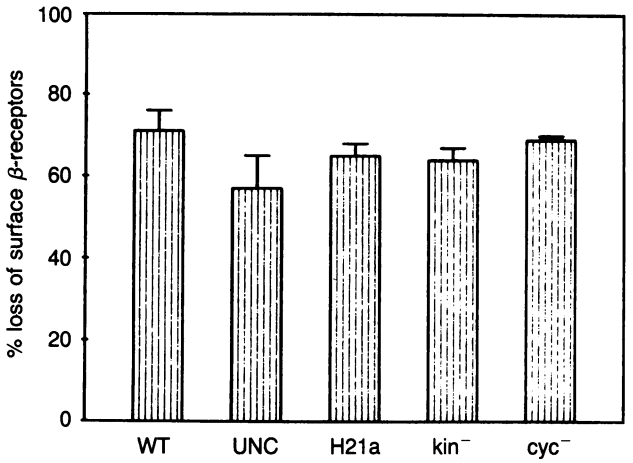

FIG. 3. Loss of surface β-adrenergic receptors after 15-min incubation with (−)-isoproterenol with wild-type and variant S49 cells. Wild-type and variant S49 cells were incubated with (−)-isoproterenol for 15 min, then washed, and specific binding of 5 nM CGP (final concentration) was assessed. Data shown are mean ± SEM of results obtained in 3 separate experiments. Bars represent wild-type (WT), UNC, H21a, kin⁻, and cyc⁻ S49 cells.

receptors and the time-dependent change in agonist affinity are a manifestation of the same process. Our current and previous results in S49 cells show that neither the time-dependent decrease in agonist affinity of intact cells nor internalization [loss of CGP or iodocyanopindolol binding sites (assayed at 4°C) from the cell surface] requires the participation of several distal components in the receptor response pathway. Further studies will be required to determine whether internalization of β-adrenergic receptors, as assessed using the approaches described here, is qualitatively different than internalization observed for receptors for other hormones, growth factors, and transport proteins.

Based on abundant data in other membrane receptor systems, we anticipated that loss of surface receptors might be the key event in the pathway leading to receptor down-regulation. The current results indicate, however, that this loss of surface receptors *per se* is not sufficient to identify a population of receptors to be down-regulated in S49 cells. In this system, the rapidity and degree to which cells down-regulate receptors relates to cellular expression of N_s activity. This implies that receptor down-regulation involves an N_s-mediated targeting of receptors, perhaps via the introduction of an N_s-promoted covalent modification in the internalized receptor, a differential segregation of receptors after interaction with N_s, or a change in the ability of internalized receptors to recycle back to the surface. The similar rates for receptor recovery from down-regulation in UNC, H21a, and wild-type S49 cells indicate that this latter explanation is not likely. Thus, N_s seems not to be involved in receptor recycling but only in the development of down-regulation. It is intriguing that cells with altered R–N_s interaction (cyc⁻, UNC) show blunted down-regulation, whereas cells with altered N_s–C interaction (H21a) down-regulate more rapidly than do wild-type S49 cells. This suggests that stoichiometric aspects and/or duration of R–N_s interactions after exposure to agonist are key determinants of down-regulation. Events distal to adenylate cyclase, in particular cAMP-dependent protein phosphorylation (which is absent in kin⁻ cells), are apparently not required for internalization, down-regulation, or desensitization in S49 cells (10, 19, 26). This is in contract to the cAMP-mediated desensitization and phosphorylation of β-adrenergic receptors reported in studies with turkey erythrocytes (32).

Analysis of the data on down-regulation of β-adrenergic receptors in wild-type S49 cells provides some information as to the cellular processes mediating changes in receptor expression. Under basal conditions, β-adrenergic receptors on wild-type S49 cells have a half-life of ≈30 hr (33). The current studies show that detectable receptors are lost at a substantially faster rate after agonist treatment (Fig. 4, *Left*). Moreover, receptor recovery from down-regulation is also substantially faster than the rate at which new receptors appear on these cells under basal conditions (Fig. 4, *Right*; refs. 33 and 34). Thus, down-regulation appears to result from an enhanced rate of receptor disappearance rather than a slower rate of receptor appearance. It is unclear whether the enhanced rate of receptor appearance during recovery from down-regulation is a consequence of accelerated receptor synthesis or mobilization of a retained but undetectable receptor pool.

The results in the S49 system offer evidence that down-

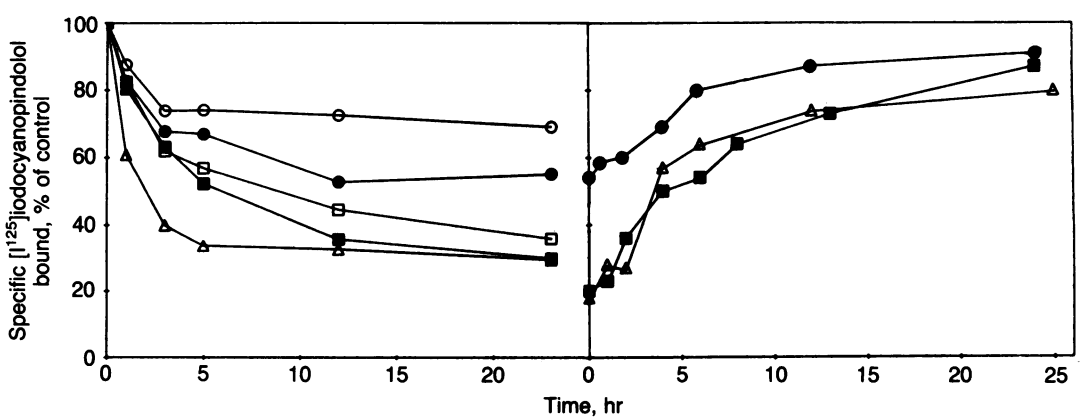

FIG. 4. Kinetics of (−)-isoproterenol-stimulated down-regulation of β-adrenergic receptors of intact S49 lymphoma cells. (*Left*) Wild-type (■) and variant S49 cells (cyc⁻, ○; UNC, ●; kin⁻, □; and H21a, △) were incubated at 37°C in DME medium/horse serum in a humidified incubator at 37°C (90% air/10% CO_2) in the presence or absence of 1 μM (−)-isoproterenol. At the indicated times, cells were washed, resuspended, and incubated with 120 pM iodocyanopindolol at 37°C for 60 min. As shown in Fig. 1 (*Lower*), loss in [^{125}I]iodocyanopindolol binding sites reflects a decrease in B_{max} and not a change in K_d; similar alterations in B_{max} and not K_d were observed for variant cells (data not shown). (*Right*) Wild-type (■), UNC (●), and H21a (△) cells were incubated with 1 μM (−)-isoproterenol for 15–18 hr at 37°C, washed, and reincubated in fresh medium for various times. Binding was assessed using iodocyanopindolol in assays conducted at 37°C for 60 min. Coefficient of variation at each time point was, in general, <10%. Data shown are mean values of results obtained from 2–3 separate experiments in which cell types were compared in parallel incubations.

regulation of β-adrenergic receptors may be regulated quite differently than that of other classes of cell-surface receptors. For many other types of membrane receptors, an agonist-promoted loss of surface sites and subsequent down regulation are closely coupled events (1–3, 24). It is tempting to speculate that receptors that involve a discrete coupling protein, such as N_s, may use this protein to trigger not only rapidly responding second messenger systems, but also delayed events that regulate receptor expression on the cell surface.

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