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Repetitive Endocytosis and Recycling of the β_2-Adrenergic Receptor during Agonist-Induced Steady State Redistribution

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SUMMARY

The human β_2-adrenergic receptor (β_2AR) rapidly internalizes after binding agonist, resulting in a dramatic redistribution of receptors from the plasma membrane and into endocytic vesicles. We sought to determine whether intracellular receptors constitute a static pool or represent a fraction of dynamically internalizing and recycling receptors. Using cells expressing a β_2AR with an epitope tag at its amino-terminal ectodomain, changes in surface receptor levels were measured by flow cytometry and radioligand binding assays. The addition of a saturating level of a strong agonist (isoproterenol) caused the endocytosis of receptors with first-order kinetics (k_e for naive cells, 0.222 min^{-1}). After 10 min, the level of surface receptors remained stable at ~20% that of untreated cells, even though endocytosis continued with similar kinetics (k_e for pretreated cells, 0.258 min^{-1}), suggesting that internalized receptors were cycling in steady state with surface receptors. This prediction was confirmed directly by showing that internalized β_2ARs recycled to the cell surface in the continued presence of agonist. The calculated transit times (1/k) in the presence of isoproterenol were 3.9 min for endocytosis and 11.2 min for recycling. The endocytic rate constant and the steady state redistribution to the internal pool were much lower after treatment with the partial agonist albuterol, suggesting a correlation between the efficiency of endocytosis and that of receptor coupling to the downstream signal transduction pathway. These findings indicate that in the presence of agonist, β_2ARs are in a dynamic steady state between the plasma membrane and endosomes that is regulated principally by agonist efficacy.

A critical feature of many G protein-coupled receptors is their ability to desensitize after agonist binding. The human β_2AR is a model system for which desensitization mechanisms have been studied extensively (for review, see Ref. 1). Agonist binding of β_2ARs results in the activation of adenylyl cyclase via G_s (a heterotrimeric stimulatory G protein) and subsequent elevation of intracellular cAMP levels. Desensitization of receptors then occurs rapidly via several distinct mechanisms. Receptors are phosphorylated by at least three kinases (protein kinases A and C and βARK) and are uncoupled functionally from G_s. Concomitant with phosphorylation, receptors internalize through clathrin-coated pits, resulting in a redistribution of receptors from the plasma membrane and into early endosomes. This compartment has been defined by the colocalization of internalized β_2ARs with the endosome markers rab5 and transferrin receptor (2, 3). Removal of agonist results in the redistribution of receptors back to a predominantly cell-surface localization (4). Although these mechanisms have been examined mainly using transfected, immortalized cell lines, similar events have been observed in primary cultures of human airway smooth muscle (5, 6), bovine and human bronchial epithelium (7, 8), human lung mast cells (9), and human alveolar macrophages (8).

The ligand-activated endocytosis of G protein-coupled receptors is a widely observed phenomenon, in both cultured cells and several *in vivo* systems (10–12). Extensive study of β_2ARs suggests that receptor endocytosis serves several distinct functions. Passage through the endocytic compartment has been proposed as the mechanism by which β_2ARs are resensitized, probably by dephosphorylation, before their return to the cell surface (13, 14). In addition, the endocytosis of β_2ARs may lead to down-regulation, defined as the mate-

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ABBREVIATIONS: AR, adrenergic receptor; βARK, β_2-adrenergic receptor kinase; CMF-PBS, Ca^{2+} and Mg^{2+}-free phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; HA, influenza virus hemagglutinin epitope; ISO, isoproterenol; k_e, first-order endocytic rate constant; mAb, monoclonal antibody; PBS, phosphate-buffered saline; DMEM-H, Dulbecco's modified Eagle's medium and 20 mM HEPES, pH 7.4; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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rial loss of total receptors over a period of hours of agonist exposure. Down-regulation may result from sorting of internalized β_2ARs from endosomes to lysosomes for degradation, although there has been no direct evidence for this (10). The fate of internalized β_2ARs thus is either resensitization and recycling or down-regulation.

It is unclear how agonist binding changes the steady state distribution of β_2ARs between endosomes and the plasma membrane. In one model ("static"), agonists would initiate the endocytosis of only a fraction of liganded surface receptors and maintain these receptors within the intracellular pool. Because β_2ARs seem to internalize by way of the constitutive endocytic pathway (3), a static model would require both a conditional endocytic mechanism, so that all liganded surface receptors would not be internalized, and an agonist-regulated recycling mechanism, to keep β_2ARs from returning to the surface without affecting the traffic of recycling receptors such as transferrin receptors. A "dynamic" model predicts that agonist binding would induce endocytosis and that agonist-bound β_2ARs would enter the constitutive endocytic pathway and recycle to the cell surface even in the continued presence of agonist. The distribution of receptors between the plasma membrane and endosomes would then be a function of the rates of endocytosis and recycling.

Previous studies of β_2AR trafficking have measured agonist-stimulated receptor endocytosis and recycling by first removing the agonist to allow the use of hydrophilic radioligand antagonists for quantification of surface receptors (15, 16). However, distinguishing between dynamic and static models requires the measurement of surface β_2ARs in the continued presence of agonist. Other researchers have provided evidence in favor of a dynamic model (17, 18); however, the assays used precluded adequate quantitative analyses. In the present study, we used a cell line overexpressing β_2ARs that have epitope tags (HA) on their amino-terminal ectodomains (2, 3). The disappearance of receptors from the cell surface and their reappearance can be quantified by the binding of an anti-HA antibody followed by flow cytometry. The distinct advantage of this approach is that the rate constants of receptor endocytosis can be measured even after most β_2ARs have been internalized by pretreatment with agonist. Thus, the kinetics of endocytosis and recycling in both naive cells and in cells pretreated with agonist can be determined, and the effects of downstream effector systems on receptor trafficking can be evaluated.

Experimental Procedures

Materials. [^{3}H]CGP-12177 (44 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). Mouse monoclonal IgG against the HA tag (mAb 12CA5) was purchased from Berkeley Antibody (Berkeley, CA) or Boehinger-Mannheim Biochemicals (Indianapolis, IN). Goat anti-mouse FITC/IgG was purchased from Molecular Probes (Eugene, OR), and nonspecific mouse IgG was from Coulter Cytometry (Hialeah, FL). All other reagents were from Sigma Chemical (St. Louis, MO) unless otherwise specified.

Cell growth and harvest. The 12β6 cell line was derived by stable transfection of HEK 293 cells with a cDNA encoding a β_2AR with the HA tag on its amino-terminal ectodomain (2) and was a generous gift of B. Kobilka (Stanford University, Stanford, CA). β_2ARs are expressed at a level of ~300,000/cell in this line (2, 3). The cells were cultured in 100-mm dishes with DMEM containing 10% fetal bovine serum and 250 μg/ml G-418 (GIBCO BRL, Gaithersburg, MD). After growth to 50–60% confluence, the cells were harvested by trituration in ice-cold DMEM-H and then washed twice by resuspension in ice-cold DMEM-H and centrifugation at 500 × g for 5 min.

Antibody binding and flow cytometry. 12β6 cells were suspended in ice-cold Dulbecco's PBS at a concentration of 10^6 cells/ml with 15 μg/ml mAb 12CA5 or a nonspecific mouse IgG to define background fluorescence. After 60 min on ice, the cells were centrifuged at 500 × g for 5 min and washed twice by resuspension in ice-cold PBS and centrifugation at 500 × g. The cells were labeled with 15 μg/ml FITC/anti-mouse IgG for 60 min on ice and then washed and fixed with 1% paraformaldehyde in PBS. Surface fluorescence was quantified by flow cytometry on an Epics Profile I (Coulter Cytometry) with three fluorescence channels using an argon laser tuned to 488 nm. Green (FL1) fluorescent events were accumulated on a four-decade logarithmic scale. Background fluorescence with nonspecific IgG was <10% of the fluorescence level with mAb 12CA5 and was subtracted to obtain a specific fluorescence level.

Radioligand binding assay of β_2AR endocytosis. This was done essentially as described previously (3). Briefly, triplicate portions of 10^5 intact 12β6 cells (~20 μg total protein) were suspended in 0.5 ml of DMEM-H with 4 nM [^{3}H]CGP-12177 with or without 3 μM alprenolol (to define nonspecific binding) and incubated on ice for 16 hr. The suspensions were filtered through glass-fiber filters (GF/C, Whatman, Maidstone, UK), and the bound radioactivity then counted by scintillation spectroscopy. Protein determinations were performed using the Coomassie Plus reagent (Pierce Chemical, Rockford, IL).

Assay of β_2AR endocytosis using flow cytometry. Two internalization protocols were followed: 1) 12β6 cells were first incubated with mAb 12CA5 (15 μg/10^6 cells/ml) on ice for 60 min. After being washed twice in DMEM-H, the cells were diluted 5-fold into 37° medium containing 5 μM ISO. Aliquots were taken at intervals and swiftly chilled by 10-fold dilution into ice-cold CMF-PBS. After centrifugation, the cells were resuspended in ice-cold CMF-PBS, labeled with FITC/IgG, and then analyzed by flow cytometry. 2) 12β6 cells were first treated with 5 μM ISO in DMEM-H for 15 min at 37° and then chilled and incubated with mAb 12CA5 (15 μg/10^6 cells/ml) on ice for 60 min. The cells were washed twice and then returned rapidly to 37° in DMEM-H with 5 μM ISO, sampled at short intervals, and chilled quickly as described above. The amount of mAb 12CA5 remaining on the surface was measured by binding with FITC/anti-mouse IgG and flow cytometry. To measure the dissociation of mAb 12CA5 from the cell surface, cells were incubated in DMEM-H without glucose and with 10 mM 2-deoxyglucose and 10 mM Na azide (buffer B) at 37° for 15 min before the addition of 5 μM ISO for 15 min. The cells were then treated exactly as described above, except that the 37° incubation in the presence of 5 μM ISO was done in buffer B rather than DMEM-H.

Receptor recycling. The rate at which internalized receptors returned to the cell surface was measured using radioligand binding and flow cytometry. 1) For radioligand binding, 12β6 cells were exposed to 5 μM ISO at 37° for 15 min, chilled and washed three times in DMEM-H, and finally resuspended in 37° DMEM-H with 4 nM [^{3}H]CGP-12177. Samples were withdrawn at intervals, pipetted into chilled tubes in an ice-water bath, and kept on ice for 16 hr. Bound ligand was measured by filtration through glass-fiber filters followed by scintillation spectroscopy. Nonspecific binding of radioligand was determined by additional incubations in the presence of 3 μM alprenolol. 2) For flow cytometry, 12β6 cells were harvested and then exposed to 5 μM ISO for 15 min at 37° and then chilled to 0° and treated with pronase (12.5 μg/ml) for 15 min to remove surface HAs. Under these conditions, 80–95% of remaining cell surface HA was removed as assessed by mAb 12CA5 binding and flow cytometry. Pronase was inactivated by the addition of 4-(2-aminoethyl)benzenesulfonyl fluoride to 0.2 mM and horse serum to 10%. The cells were then washed once with DMEM + 10% fetal bovine serum, and once with DMEM-H before resuspension in DMEM-H at 37° in the presence of 5 μM ISO. Samples were taken at brief intervals and diluted

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into 10 volumes of ice-cold CMF-PBS. Surface HAs were quantified by staining with mAb 12CA5 and FITC/IgG, followed by flow cytometry.

Data analysis. The change in concentration of receptor at the surface per unit time after the addition of agonist is given by:

$$\frac{dR_s}{dt} = -k_e R_s + k_r R_i \quad (1)$$

where R_s is the fraction of receptors on the cell surface, R_i is the fraction of receptors inside the cell, k_e is the endocytic rate constant, and k_r is the recycling rate constant. When starting with 100% of receptors at the surface, the concentration of receptors at the surface at subsequent times after agonist addition is obtained by integration of equation 1 with the boundary conditions R_s at t = 0 defined as R_{s0}, and R_s at t to infinity is defined as $R_s\infty$. The solution is:

$$R_s = (R_{s0} - R_s\infty)e^{-(k_e + k_r)t} + R_s\infty \quad (2)$$

Note that:

$$R_s\infty = R_{s0}\frac{k_r}{k_r + k_e} \quad (3)$$

By substituting eq. 2 into eq. 3 and rearranging, we obtain the following:

$$\frac{R_s}{R_{s0}} = \frac{k_r(1 - e^{-(k_e + k_r)t})}{k_e + k_r} + e^{-(k_e + k_r)t} \quad (4)$$

This equation is identical to one derived by Holman *et al.* (19) to describe the trafficking of the glucose transporter GLUT-4 between endosomes and the plasma membrane in transfected fibroblasts in response to insulin. The model assumes that in stimulated cells, GLUT-4 is in steady state between only those two cellular pools (two-pool model).

Receptor recycling curves also make use of the first equation but with k_e set to zero (in the absence of an agonist) and with the initial conditions that $R_s = R_s\infty$ and $R_i = (R_{s0} - R_s\infty)$. Then, R_i as a function of time is given by:

$$R_i = (R_{s0} - R_s\infty)e^{-k_r t} \quad (5)$$

Experimentally, we obtain:

$$R_i = (R_s\infty - R_{st}) \quad (6)$$

where $R_s\infty$ is the concentration of receptors at the surface long after agonist withdrawal, and R_{st} is the concentration of surface receptors at time t. All curve-fitting was done using the computer program GraphPAD Prizm (version 1.03; San Diego, CA). Goodness of fit was evaluated using a runs test to determine the probability that the experimental data points are randomly distributed above and below the curve. Results of the runs tests are expressed as probability values, where $p > 0.05$ is considered not significant. In addition to first-order rate constants, we derived transit times, defined as the length of time required for surface receptors to complete a step of endocytosis ($1/k_e$) or recycling ($1/k_r$).

Results

Measurement of surface β_2ARs using flow cytometry. This approach was necessary to measure the surface levels of β_2ARs in the continued presence of agonist. The effect of mAb 12CA5 binding on agonist-evoked endocytosis of HA/β_2AR was assessed by radioligand binding. ISO caused a time-dependent reduction in the specific surface binding of the hydrophilic radioligand antagonist [^{3}H]CGP-12177 that reached a plateau within 5 min of agonist exposure. The extent and kinetics of endocytosis were identical regardless of whether the cells were incubated with mAb 12CA5 or with nonspecific mouse IgG (Fig. 1). These kinetics were consistent with our previous findings (3) and show that binding the HA with mAb 12CA5 did not significantly affect the degree or time course of ISO-induced β_2AR endocytosis. Binding of mAb 12CA5, in the absence of agonist, did not itself cause β_2AR endocytosis (Fig. 1), which is in agreement with previous findings (18).

Endocytosis of β_2ARs. Receptor endocytosis first was measured using 12β6 cells that had never been exposed to agonist (naive cells). After incubation with mAb 12CA5 at 0°, the cells were suspended at 37° with 5 μM ISO and sampled at short intervals to quantify remaining surface receptors by flow cytometry. β_2ARs rapidly internalized for several minutes before endocytosis apparently stopped after >80% of the receptors were removed from the cell surface (Fig. 2A, ■). To determine whether this new distribution reflected a dynamic state or a static one, 12β6 cells were first incubated with 5 μM ISO at 37° to achieve maximum endocytosis (>80% of receptors internalized) and then chilled to 0°, and the remaining surface receptors were bound to mAb 12CA5. The cells were then incubated at 37° with 5 μM ISO and sampled at intervals to quantify surface receptors by flow cytometry. The shape of the curve and the extent of endocytosis are quite similar to those of naive cells (Fig. 2A, □). In both experiments, the total amount of bound mAb 12CA5 remained relatively constant between 5 and 20 min after the start of internalization assays, suggesting that antibody remained bound to receptors during endocytosis and recycling and was not appreciably degraded by intracellular proteases. The loss of surface mAb 12CA5 between 0 and 5 min was due almost entirely to receptor endocytosis and not to dissociation of the antibody from the epitope tag, because there was very little loss of surface mAb 12CA5 in the absence of endocytosis (Fig. 2A, △). The initial rate constants for endocytosis (k_e) were estimated by plotting the natural logarithms of the fractions remaining on the cell surface as a function of time after agonist addition over the first 4 min of internalization, an

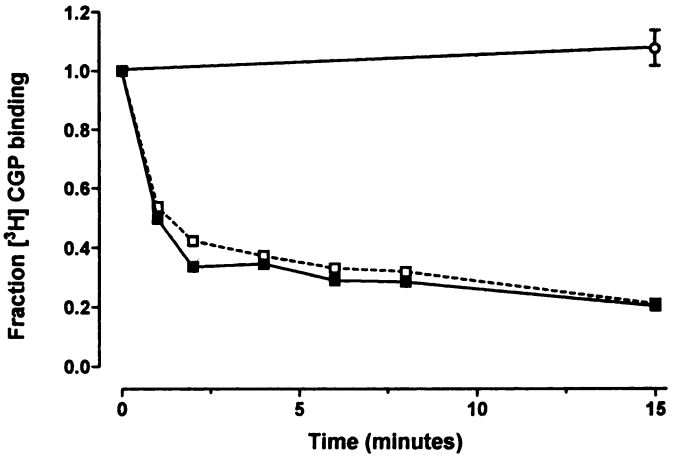

Fig. 1. The effect of mAb 12CA5 binding on agonist-induced β_2AR endocytosis. 12β6 cells suspended in ice-cold DMEM-H at 10^6 cells/ml were incubated with either 15 μg/ml mAb 12CA5 or nonspecific IgG on ice for 60 min. The cells were then diluted into DMEM-H at 37° with (□, ■) or without (○) 5 μM ISO and sampled at various times by rapid chilling. After washing, the cells were incubated with [^{3}H]CGP-12177 to determine the level of surface β_2AR. □, mAb 12CA5 with ISO; ■, control IgG with ISO; ○, mAb 12CA5 without ISO. Internalization curves are representative.

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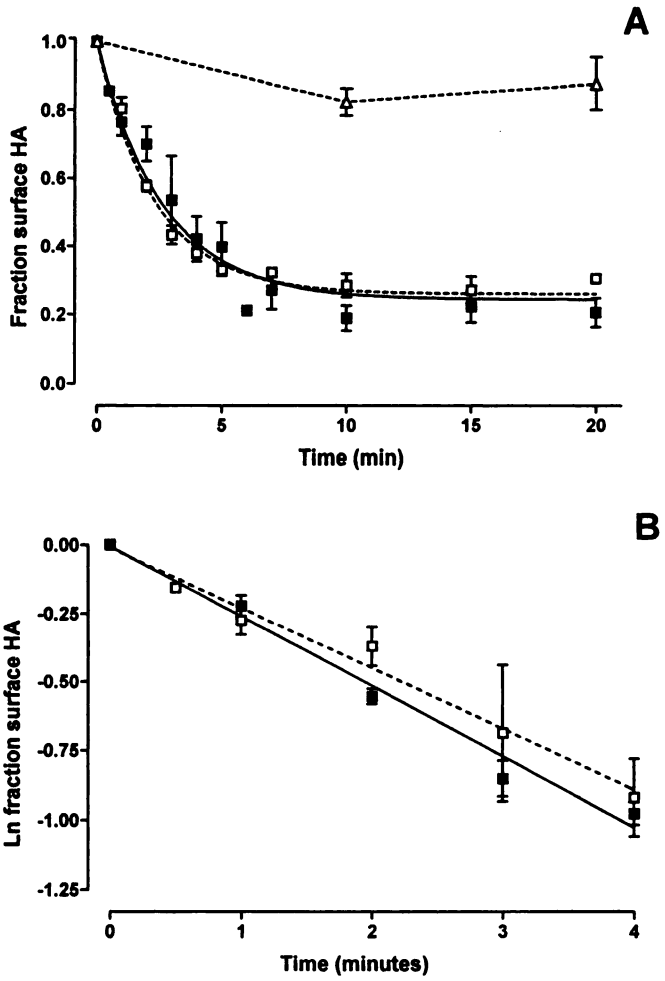

Fig. 2. Endocytosis of β_2AR. ■, 12β6 cells were incubated at 0° for 60 min with mAb 12CA5 or nonspecific IgG and then quickly warmed to 37° in DMEM-H containing 5 μM ISO. Aliquots then were removed for analyses by flow cytometry. □, 12β6 cells were first incubated at 37° in DMEM-H with 5 μM ISO for 20 min and then chilled and incubated with mAb 12CA5 or nonspecific IgG at 0° for 60 min. The cells were quickly warmed to 37° in DMEM-H with 5 μM ISO, and aliquots were removed for analysis by flow cytometry. △, Cells were treated the same way as for □, except that all incubations occurred in the absence of glucose and the presence of 10 mM 5-deoxyglucose and 10 mM Na azide. A, Complete time course is plotted, and the curves were fitted to equation 4 (see Experimental Procedures). B, Data from 0–4 min of A were converted to natural logarithms and plotted on linear axes. Linear regression was used to determine the slopes of these curves. A runs test to assess linearity gave values of $p = 0.175$ for naive cells and $p = 0.205$ for ISO-pretreated cells. *Error bars*, standard error. ■, Naive cells; □, ISO-pretreated cells.

interval during which antibody bound to receptors has not yet recycled (Fig. 2B). Linear regression analysis of these curves yielded a k_e value of 0.222 ± 0.027 min^{-1} (transit time, 4.5 min) for naive cells and of 0.258 ± 0.013 min^{-1} (transit time, 3.9 min) for ISO-pretreated cells. These rate constants can be used in curve-fitting of the data; however, because recycling is occurring at the same time as endocytosis, rate constants are needed for both processes. Before fitting the curves in Fig. 2, we therefore sought to determine the recycling rate constant.

Measurements of β_2AR recycling rates after removal of agonist. The quantification of β_2AR trafficking requires determining rate constants for each step of receptor transfer from one cellular compartment to another. One step that can be quantified in isolation from other steps is the recycling of internalized β_2ARs back to the cell surface after removal of the agonist (15, 16). After the withdrawal of the agonist, there is little or no receptor endocytosis (data not shown); thus, the effects of endocytosis during the recycling assay are minimal. The rate constant for receptor recycling (k_r) was determined by treating cells with agonist to achieve maximal endocytosis (70–80% of receptors internalized) and then measuring the level of β_2ARs remaining intracellular as a function of time after agonist removal (Fig. 3). Modeling of this curve using equation 5 indicates that β_2ARs recycled to the cell surface with first-order kinetics, giving a rate constant (k_r) of 0.091 ± 0.0143 ($p = 0.805$, runs test). These data yield a half-time for recycling ($0.693/k_r$) of 7.9 min and a transit time ($1/k_r$) of 11.2 min.

Curve fitting the internalization and recycling data. The initial rate constants for β_2AR endocytosis determined in Fig. 2B and the recycling rate constant (Fig. 3) were used as initial values for curve fitting the data for ISO-pretreated cells in Fig. 2A. For naive cells, the experimentally determined k_r was fixed at 0.091, as determined in Fig. 3 for recycling in the absence of agonist. The equation we used (equation 4) describes the trafficking of integral membrane proteins between two cellular pools, the plasma membrane and an intracellular compartment (19, 20). Curve fitting was evaluated using a runs test, which gave values of $p = 0.582$ for ISO pretreated cells and $p = 0.842$ for naive cells (neither value is significant). The calculated k_e was 0.288 ± 0.017 min^{-1} for naive cells and 0.317 ± 0.017 min^{-1} for ISO-pretreated cells (Table 1). Although these values of k_e are slightly higher than those derived from initial rates, they are quite close to each other, indicating that prior agonist exposure had little effect on the endocytic rate constant of β_2ARs.

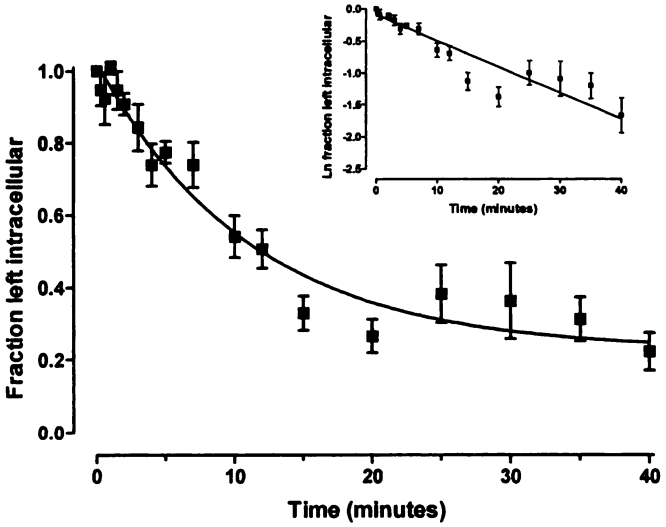

Fig. 3. Recycling of β_2ARs after withdrawal of agonist. 12β6 cells were suspended in DMEM-H at 37° for 15 min in the presence of 5 μM ISO. Cells were chilled, washed three times, and resuspended in DMEM-H at 37° with [^{3}H]CGP-12177 (4 nM). Aliquots were removed at various time intervals, chilled, kept overnight at 0°, and filtered. Each point is the average of three or five determinations. *Error bars*, standard error. The plot of receptors left intracellular as a function of time was fitted to equation 5 (see Experimental Procedures). *Inset*, fraction of β_2ARs left inside the cell was converted to natural logarithms and plotted on a linear scale.

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TABLE 1
Summary of β_2AR trafficking kinetics and distribution

Pretreatment	Agonist	No. of measurements	k_e (direct)[a]	k_e (calculated)[b]	R_s^{∞}[c]	k_r[d]
None	ISO	6	0.222[e,f] (0.027)	0.288[g] (0.017)	0.183[h,i] (0.030)	0.091 (0.014)
ISO	ISO	7	0.258[e] (0.013)	0.317[g] (0.017)	0.262[h] (0.019)	0.113 (0.015)
None	ALB	6	0.037[f] (0.005)	0.040 (0.002)	0.718[h] (0.027)	0.091 (0.014)

Numbers in parentheses are standard errors.
[a] min^{-1}, initial rates, from slopes of logarithmic plots in Figs. 2B and 4B.
[b] min^{-1}, calculated from fitting of curves in Figs. 2A and 4A, with k_r fixed at 0.091 min^{-1} for cells with no pretreatment.
[c] Fraction of β_2ARs remaining on the cell surface at steady state.
[d] min^{-1}, determined in Fig. 3 for cells with no pretreatment; derived by curve-fitting, allowing both k_e and k_r to vary for ISO-pretreated cells.
[e] $p = 0.2334$.
[f] $p < 0.001$.
[g] $p = 0.322$.
[h] $p < 0.001$.
[i] $p = 0.157$.

Curve fitting also yielded a unique value for k_r in ISO-treated cells of 0.113 ± 0.015 min^{-1}. This value is very similar to that determined using [^{3}H]CGP-12177 binding on agonist withdrawn cells (0.091 min^{-1}).

Receptor endocytosis driven by a partial agonist. The very small difference in the endocytic rate constants between agonist-pretreated and naive cells suggests that downstream signal transduction events play little, if any, role in regulating β_2AR endocytosis. We thus hypothesized that β_2AR endocytosis is governed principally by the conversion of receptor to its activated form (R^*) after agonist binding. To test this hypothesis, we measured the rate of β_2AR internalization caused by the partial agonist albuterol, which fully activates adenylyl cyclase in 12β6 cells due to the high levels of receptors, even though the coupling efficiency (as an index of ability to drive receptor conformation to R*) is only 12% that of ISO.[2] We performed an internalization assay in which naive cells were exposed to 5 μM albuterol for varying times, followed by [^{3}H]CGP-12177 binding to measure the loss of surface β_2ARs (Fig. 4A). The initial rate of receptor endocytosis was 0.037 ± 0.0054 min^{-1} (Fig. 4B). When curve fitting was performed with k_r fixed at 0.091 min^{-1}, a k_e of 0.040 ± 0.002 min^{-1} was obtained ($p = 0.320$). The steady state distribution of β_2ARs in the continued presence of agonist was 71.8% on the cell surface (Table 1).

β_2AR recycling in the presence of agonist. The endocytosis studies suggested that after 10 min in the presence of agonist, β_2ARs traffic in a dynamic steady state between the cell surface and intracellular compartments. To show directly that receptor recycling occurs in the continued presence of agonist, receptors were internalized with agonist, and the remaining surface HAs were removed by digestion with pronase. This treatment did not affect the binding of [^{3}H]CGP-12177 or the extent of endocytosis induced by ISO in naive cells as measured by radioligand binding (data not shown). The treated cells were then incubated at 37° and sampled at short intervals to follow the rate at which intracellular receptor HAs (which were protected from pronase treatment) returned to the surface. The curve in Fig. 5 clearly indicates that receptors returned to the cell surface in the presence of agonist and that a steady state redistribution of receptors between the surface and intracellular compartments was achieved after 10 min. The pronase treatment may have perturbed trafficking to some extent, as suggested by the "overshoot" of the curve between 3 and 5 min after warming to 37°. Thus, this method does not provide a quantitative estimate of receptor recycling in the presence of agonist but rather a qualitative demonstration of its occurrence.

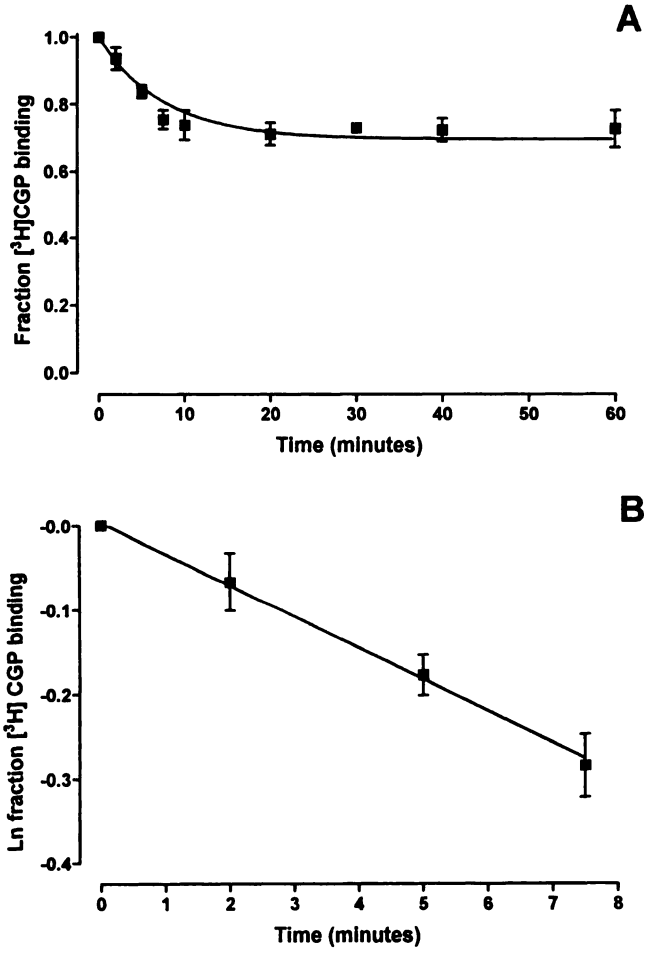

Fig. 4. Internalization driven by the partial agonist albuterol. 12β6 cells were harvested and then suspended in DMEM-H at 37° with 5 μM albuterol. Aliquots were withdrawn at intervals and rapidly chilled for binding to [^{3}H]CGP-12177 as described in Experimental Procedures. A, Complete time course is plotted and fitted to equation 5 ($p = 0.5$). B, Points from 0–7.5 min were converted to natural logarithms and plotted on a linear scale ($p = 0.496$).

[2] B. Whaley, C. Allal, A. Siebold, R. Clark, and R. Barber. Submitted for publication.

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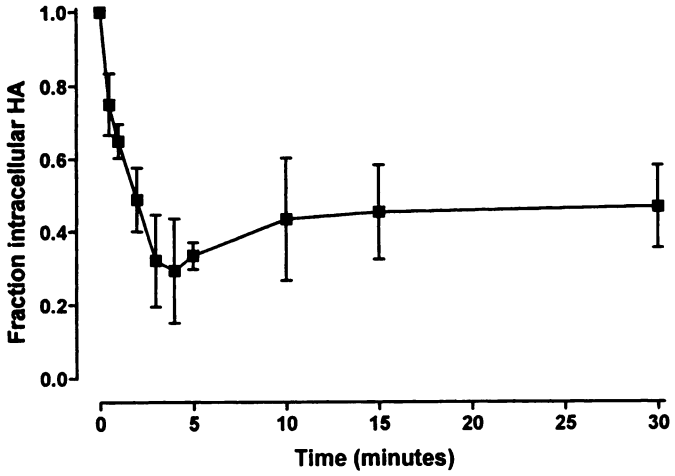

Fig. 5. Recycling of the β_2AR in the continued presence of agonist. 12β6 cells were exposed to 5 μM ISO at 37° for 10 min and then chilled to 0° and treated with pronase (12.5 μg/ml; 15 min). After removal of enzyme, the cells were resuspended in DMEM-H at 37° with 5 μM ISO, sampled at intervals thereafter, chilled, and processed for flow cytometry to determine the level of HA epitopes that had returned to the surface. *Points*, average of three determinations reflecting the net increase of fluorescence over the zero time point. *Error bars*, standard error.

Discussion

This study shows directly that β_2ARs internalize and recycle repetitively in the presence of agonist, behaving under these conditions as receptors that constitutively internalize and recycle such as the low density lipoprotein and transferrin receptors (21). We also have determined the rate constants for these processes, which suggest that agonist regulates β_2AR endocytosis primarily by the binding and activation of receptor *per se*.

Our measurements of endocytosis and recycling were done within short time courses of agonist exposure, before there was significant receptor down-regulation (data not shown; Ref. 3). The slow rate of down-regulation in the 12β6 line greatly simplifies the measurement of receptor endocytosis and recycling and permits the analysis of data using relatively simple mathematical models. Also, the use of flow cytometry allows the measurement of receptor endocytosis in the continued presence of agonist. This is a valid method to measure cell surface receptors because binding of antibody itself did not cause receptor endocytosis and did not detectably influence receptor endocytosis induced by agonist (Fig. 1).

The experimentally determined rate constants for ISO-induced endocytosis (k_e) of β_2ARs was 0.222 min^{-1} for naive cells and 0.258 min^{-1} in those cells that had been exposed previously to ISO (Fig. 2B and Table 1). These values are not significantly different, suggesting that preexposure to agonist, which greatly increases cAMP levels, has little effect on the rates of β_2AR endocytosis. This is consistent with a previous finding that β_2AR endocytosis in response to agonist occurs normally in mutant S49 lymphoma cells lacking G_s (*cyc*$^-$) or protein kinase A (*kin*$^-$) (22). In contrast, the experimentally determined endocytic rate constant for naive cells in response to the partial agonist albuterol is only approximately one-sixth that of naive cells treated with ISO (Fig. 4 and Table 1). This finding is significant because albuterol causes full activation of adenylyl cyclase in 12β6 cells due to the high density of β_2ARs despite a coupling efficiency that is only 12% that of ISO.[2] This observation supports a model in which the rate of β_2AR endocytosis is proportional to the efficiency with which β_2AR couples to its effector system as a result of agonist binding. This idea is consistent with recent findings that proteins involved in the phosphorylation and uncoupling of agonist-occupied β_2ARs may be connected with the regulation of surface receptor levels. Agonist-occupied β_2ARs are phosphorylated on their carboxyl-termini by βARK-1 (23), followed by interaction of the phosphorylated receptors with β-arrestin (24). The endocytosis defect of a β_2AR mutant (Y326A) is apparently rescued by overexpression of either βARK-1 (25) or β-arrestin (26), suggesting a role for these proteins in β_2AR endocytosis. Agonist-induced binding of these proteins may be a key step in controlling the trafficking of β_2ARs. However, it is not clear whether endocytosis alone is regulated, or if there also are influences on receptor recycling. Detailed kinetic studies of β_2AR endocytosis and recycling under conditions of varying βARK-1, β-arrestin, and phosphatase activities are needed to determine what molecular steps of receptor trafficking are regulated.

Our determination of endocytic rate constants for β_2ARs may be compared with several earlier studies. Ligand-induced β_2AR endocytosis in A431 cells exhibits a k_e of 0.03 min^{-1} and a k_r of 0.06 min^{-1}, with a maximum extent of ~25% of receptors internalized (27). In contrast, wild-type S49 lymphoma cells seem to internalize receptors with a k_e of 0.73 min^{-1} and recycle with a rate constant of 0.042 min^{-1} (22). These endocytic rate constants are quite dissimilar to what we have determined and may be accounted for by differences in the cell types being studied or in the types of internalization assays used. Our rate constants are more in line with those determined for some other receptor model systems. Among G protein-coupled receptors, muscarinic acetylcholine receptors show an endocytic rate constant of ~0.12 min^{-1} in NG108-15 cells (28), and glucagon-like peptide-1 receptors internalize with a rate constant in the range of 0.20–0.30 min^{-1} (29). The ligand-induced endocytosis of the epidermal growth factor receptor occurs with rate constants of 0.20–0.50 min^{-1} (30, 31). All of these rate constants for ligand-induced endocytosis lie within the range of that determined for the constitutively trafficking transferrin receptor, which recently was determined to be 0.28 min^{-1} (32). This supports the idea that ligand-stimulated endocytosis occurs by the same pathways used by constitutively trafficking receptors (3).

We cannot rule out that agonist has some effect on the rate of β_2AR recycling; however, the experimentally determined value for k_r measured after the removal of agonist (0.091 min^{-1}) is quite similar to that of ISO-pretreated cells (0.113 min^{-1}). A previous study concluded that β_2AR recycling is not regulated by agonist; however, the methods used did not allow the careful determination of rate constants (17). More recently, several studies have examined the role of cAMP and the function of G_s in the trafficking of other membrane proteins. Using polarized epithelial cells, it was found that transport from the *trans*-Golgi network to the apical surface is stimulated by activators of G_s (33) and by manipulations that elevate intracellular cAMP (34–36). cAMP (through protein kinase A) also stimulates the transcytosis of polymeric IgA receptor from the basolateral domain to the apical sur-

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face, although there seems to be no effect on the recycling of receptors that have been internalized via the apical surface (35). Additional work is needed to determine whether cAMP exerts any effect on β_2AR recycling and, if so, to what extent.

It is possible that some β_2ARs recycle very slowly because we never observed the return of 100% of the receptors to the cell surface over the time course of a 60-min recycling assay (data not shown), and computer modeling suggests there may be a component of receptors that recycle very slowly under conditions of agonist removal (Fig. 3). However, first-order monophasic recycling of β_2ARs in 12β6 cells would be consistent with that observed previously for β_2ARs in S49 lymphoma cells (22). Also, monophasic recycling is observed for other G protein-coupled receptors, such as the muscarinic acetylcholine receptor, whose recycling occurs with a rate constant of 0.12 min^{-1} (28), and the glucagon-like peptide-1 receptor, which recycles with a rate constant of 0.047 min^{-1} (29). In contrast to these receptors, the constitutively trafficking transferrin receptor recycles as a two-phase exponential process, with a fast rate constant of 0.249 min^{-1} and a slow rate constant of 0.013 min^{-1} (32). Further study will be needed to determine whether other constitutively cycling receptors fundamentally differ from signal-transducing receptors in this respect.

The dynamic nature of β_2AR trafficking has important implications for receptor down-regulation. This process could involve the diversion of internalized receptors from endosomes into the degradative pathway, presumably terminating in lysosomes (10). However, the connection between β_2AR endocytosis and down-regulation is hotly debated. Certain mutations within the carboxyl-terminal cytoplasmic tail of receptor can reduce down-regulation without affecting the extent of endocytosis (37, 38), although some of these mutants are pleiotropic in their effects and show aberrant coupling to G_s. Some naturally occurring β_2AR polymorphisms with amino acid substitutions in the amino-terminal ectodomain show altered down-regulation properties (increased or greatly reduced extents) and apparently normal endocytosis kinetics (39). Conversely, a mutation of the β_2AR within the seventh transmembrane domain greatly reduces endocytosis but has little or no effect on receptor down-regulation (40). Although these findings suggest that endocytosis is not rate limiting for down-regulation, they do not rule out the possibility that down-regulation requires the occurrence of some low level of endocytosis. Our findings that β_2AR endocytosis and recycling occur continuously in the presence of agonist suggest that rapidly trafficking receptors are sorted to the degradative pathway, such that any given receptor has a small probability of being sorted with each round of endocytosis and recycling. From the information in this and previous studies, it is likely that trafficking of β_2AR *per se* is more important than cAMP-mediated effects in receptor down-regulation. In support of this idea, there is no evidence for the involvement in down-regulation of either the domain of β_2AR that is phosphorylated by protein kinase A (41) or for a significant role of protein kinase A itself (42).

In conclusion, our results provide a direct demonstration of the dynamic nature of β_2AR trafficking in the presence of agonist and an explanation for the steady state level of receptor redistribution. These studies also provide evidence for a direct link between agonist efficacy and the rate of receptor endocytosis and for a less important effect of cAMP on the rates of receptor trafficking.

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