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Specific changes in β_2-adrenoceptor trafficking kinetics and intracellular sorting during downregulation

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Abstract

Agonist-activated β_2-adrenoceptors rapidly internalize and then recycle to the cell surface, however chronic agonist eventually causes receptor downregulation. To characterize β_2-adrenoceptor trafficking kinetics and intracellular sorting during downregulation, human embryonic kidney cells expressing epitope-tagged receptors were examined by radioligand binding with (±)-[^{3}H]4-(3-tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-on hydrochloride ([^{3}H]CGP12177) and immunofluorescence microscopy. The first-order receptor recycling rate constant declined after 18 h of agonist compared with 15 min (0.05 min^{-1} vs. 0.12 min^{-1}), thus increasing the intracellular transit time (20.0 min vs. 8.3 min). There was also a reduction in the rate of receptor endocytosis and a decline in the total number of receptors. Although the intracellular receptor fraction did not increase between 15 min and 18 h of agonist, some receptors moved irreversibly into a protease-containing compartment while retaining radioligand binding activity. Our results indicate that β_2-adrenoceptor downregulation is associated principally with an increased intracellular transit time during recycling. This could promote the diversion of receptors into protease-containing compartments, where there is an irreversible commitment to downregulation prior to loss of radioligand binding activity.

Keywords: β_2-Adrenoceptor; Downregulation; Recycling; Endocytosis

1. Introduction

G-protein coupled receptors comprise a large family of cell-surface signal transducing molecules acting through heterotrimeric G-proteins to activate or inhibit intracellular effector systems. A particularly well-studied model system is the human β_2-adrenoceptor, which binds catecholamine agonists and activates adenylyl cyclase through a stimulatory G-protein, G_s. Signal transduction by β_2-adrenoceptors is attenuated by several distinct, if overlapping mechanisms (reviewed by Böhm et al., 1997). Receptor phosphorylation, which leads to uncoupling from G_s, is mediated principally by G protein-coupled receptor kinases acting on the C-terminal intracellular domains of agonist-activated receptors (Fredericks et al., 1996), but also by protein kinases A and C acting at additional sites within the third intracellular loop (Yuan et al., 1994). Phosphorylation by G protein-coupled receptor kinase is associated with receptor endocytosis into endosomes, a clathrin-mediated process occurring with rapid kinetics after agonist binding that serves to remove receptors from the cell surface (Von Zastrow and Kobilka, 1992; Moore et al., 1995; Morrison et al., 1996; Zhang et al., 1996). Receptor dephosphorylation by a membrane-bound protein phosphatase may then occur within endosomes (Pippig et al., 1995; Pitcher et al., 1995; Krueger et al., 1997), after which receptors recycle rapidly to the cell surface. With continued β-adren-

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oceptor-agonist treatment, further reduction in signaling capacity is caused by receptor downregulation, defined as a loss of total cellular radioligand-binding sites, and which occurs with much slower kinetics ($t_{1/2} > 3$ h) compared with endocytosis ($t_{1/2} \sim 2$–3 min) and recycling ($t_{1/2} \sim 7$ min) (Pittman et al., 1984; Mahan and Insel, 1986; Morrison et al., 1996).

Although there has been rapid progress in understanding β_2-adrenoceptor phosphorylation and endocytosis (Ferguson et al., 1996; Koenig and Edwardson, 1997), downregulation is not quite as well understood. While there is evidence in some cell lines that β_2-adrenoceptor mRNA levels are reduced by chronic agonist treatment, leading to reduced receptor synthesis (Collins et al., 1991; Huang et al., 1993), the effect does not appear to be great enough to account for measured reductions in receptor half-lives in the presence of agonist (Mahan and Insel, 1986). Although direct biochemical evidence for receptor degradation has been obtained for G-protein coupled receptors that downregulate rapidly after activation (Shapiro and Coughlin, 1998), and for several mammalian growth-factor receptors (Beguinot et al., 1984; Stoscheck and Carpenter, 1984; Strous et al., 1996), such evidence is lacking for G-protein coupled receptors that undergo extensive recycling and slow downregulation. One model for β_2-adrenoceptor downregulation at the cellular level is that internalized receptors move from endosomes to lysosomes for degradation. Indirect evidence that rapid endocytosis is important for downregulation is that rates of receptor endocytosis are reduced using partial agonists (Morrison et al., 1996), and the rate of downregulation is approximately proportional to agonist efficacy (Pittman et al., 1984). However, the relationship between receptor endocytosis and downregulation is complex. The properties of certain mutant β_2-adrenoceptors suggests that downregulation and rapid endocytosis are not tightly coupled. Mutation of four serines and threonines between Ser^{355} and Ser^{364} (Hausdorff et al., 1991) or the single mutation $Tyr^{326} \rightarrow Ala$ (Barak et al., 1994) reduce endocytosis without apparently affecting downregulation. These and other studies suggest either that the downregulation pathway is quickly saturated by low levels of receptor endocytosis, or that endocytosis and downregulation are unrelated. Other mutant β_2-adrenoceptors internalize normally but have blunted down-regulation in response to agonist (Campbell et al., 1990), suggesting the existence of receptor structural features that control intracellular sorting events involved in downregulation.

To determine how receptor trafficking events govern receptor downregulation, we have measured the rates of receptor recycling and endocytosis as a function of agonist exposure time, and examined intracellular sorting events related to receptor downregulation. These studies have delineated several significant events occurring at the cellular level during agonist-induced downregulation of β_2-adrenoceptors.

2. Materials and methods

2.1. Materials

The 12β6 cell line, a generous gift of B. Kobilka (Stanford, CA), was derived from human embryonic kidney cells (HEK293) by stable transfection with pBCMI12 expressing a hemagglutinin epitope-tagged β_2-adrenoceptor (Von Zastrow and Kobilka, 1992). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 250 μg/ml of G418 (Geneticin, Life Technologies, Gaithersburg, MD). The level of β_2-adrenoceptor expression in the 12β6 line is approximately 3×10^5 receptors per cell (Moore et al., 1995). (±)-[^{3}H]4-(3-tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-on hydrochloride ([^{3}H]CGP-12177) (44 Ci/mmol) and ^{125}I-diferric transferrin (1 μCi/μg) were obtained from NEN Life Sciences (Boston, MA). A mouse monoclonal immunoglobulin G (IgG) against the haemagglutinin tag (mHA.11) was purchased from Berkeley Antibody (Berkeley, CA), and goat anti-mouse IgG conjugated with fluorescein isothiocyanate (IgG-FITC) from Molecular Probes (Eugene, OR). (−)-isoproterenol was purchased from ICN Biochemicals (Costa Mesa, CA), and paraformaldehyde from Electron Microscopy Sciences (Ft. Washington, PA). All other reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified.

2.2. Determination of β_2-adrenoceptor recycling rate constants

The method is based on that of Hertel and Staehelin (1983), and was described previously (Morrison et al., 1996). 12β6 cells growing in 100 mm dishes were left untreated or incubated for varying times with 5 μM isoproterenol. The cells were washed three times with ice-cold DMEM containing 20 mM HEPES, pH 7.4 (DMEM-H), then harvested into cold DMEM-H by vigorous pipetting of DMEM-H over the monolayers. A small sample of the suspension was lysed by freeze–thaw and the protein concentration measured. The suspended cells were diluted to 50–70 μg/ml into 37°C DMEM-H with 10% fetal bovine serum containing the hydrophilic radioligand antagonist [^{3}H]CGP-12177 (4 nM), which preferentially binds surface receptors. The presence of antagonist during recycling insures that no endocytosis occurs and improves assay reproducibility that might otherwise be affected by trace carryover of agonist (Staehelin and Hertel, 1992). At varying times after dilution of the cells into the 37°C medium, 1 ml samples were removed, diluted fourfold into ice-cold DMEM-H with 4 nM [^{3}H]CGP-12177 and left on ice for 60 min to insure that binding reaches equilibrium. The cells were collected by rapid filtration through GF/C

glass fiber filters and the bound tritium quantified by scintillation spectroscopy. Of the total radioligand added to the binding mixtures less than 10% was bound, and nonspecific binding, measured by parallel incubations with 3 μM propranolol, was always less than 5% of the total binding. Recycling rate constants were calculated as described in Morrison et al. (1996). Briefly, the data were first were transformed as follows:

$$R_i = (R_s\infty - R_{st}) \quad (1)$$

where R_i is the receptor level remaining internal at times t after removal of agonist, $R_s\infty$ is the surface level of receptors after the completion of recycling ('plateau') and R_{st} is the surface level at times t after removal of agonist. The plots of R_i vs. time were modeled using the following equation (Eq. 5 from Morrison et al., 1996):

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

where R_{s0} is the number of receptors at the cell surface before the addition of agonist, and k_r is the first-order recycling rate constant. Computer curve fittings were done with GraphPad Prism (v. 2).

2.3. Measurement of β_2-adrenoceptor downregulation

12β6 cells were cultured in 24-well cluster plates coated with poly-D-lysine until 50% confluent, then groups of six wells were left untreated or treated with isoproterenol (5 μM) for 18 h, 3 h or 15 min. The monolayers were washed four times with ice-cold DMEM-H, then incubated with 6 nM [^{3}H]CGP-12177 at 4°C for 60 min in the presence of 0.05% digitonin to measure total receptors. The cells were washed twice with ice-cold DMEM-H, then solubilized with 0.1% Triton X-100 and transferred to vials for counting by scintillation spectroscopy. Binding of [^{3}H]CGP-12177 to monolayer cells was saturable with a K_d of 0.5 nM, and nonspecific binding in the presence of 3 μM propranolol was less than 5% of the total (Hall and Knoll, unpublished). There was no detectable loss of cells or reduction in cell viability after prolonged agonist exposure (data not shown). [^{3}H]CGP-12177 binding in the presence of 0.05% digitonin detected similar numbers of receptors in control cells and in cells treated for 15 min with agonist to internalize 65% of the surface receptors (Fig. 2). Thus, [^{3}H]CGP-12177 binding in the presence of digitonin can efficiently detect intracellular receptors (Rands et al., 1990).

2.4. Determination of β_2-adrenoceptor internalization rate constants

12β6 cells were cultured in 24-well cluster plates coated with poly-D-lysine until 50% confluent, then isoproterenol (5 μM) was added to triplicate wells for varying times at 37°C. At the end of the time course, the clusters were chilled on ice, and the monolayers washed four times with ice-cold DMEM-H. Surface receptor levels were quantified using [^{3}H]CGP-12177 as described above, except that digitonin was left out. The fractions of receptors left remaining on the surface were plotted vs. time after agonist addition, and the curve modeled with the following equation (Eq. 4 in Morrison et al., 1996):

$$\frac{R_s}{R_{s0}} = \frac{k_r(1 - e^{-(k_e + k_r)t})}{k_e + k_r} + e^{-(k_e + k_r)t} \quad (3)$$

where R_s is the surface fraction of receptors at times t after adding agonist, R_{s0} is the surface fraction at time 0 (set at unity), k_r is the recycling rate constant, and k_e is the endocytic rate constant. Computer curve-fitting was used to generate unique values for k_r and k_e.

2.5. Extent of β_2-adrenoceptor recycling

12β6 cells were cultured in poly-D-lysine-coated 24-well cluster plates until 50% confluent, then groups of six wells were left untreated or treated with isoproterenol (5 μM) for 18 h, 3 h or 15 min. The medium was aspirated and the cells washed quickly four times with DMEM containing 10% fetal bovine serum at 37°C. The same medium containing 6 nM [^{3}H]CGP-12177 was added back to the cells and incubation continued at 37°C for 60 min. The clusters were transferred to ice and kept there for 60 min, then washed and harvested to measure bound radioligand as described above. In some experiments, leupeptin was added to 100 μM at the start of agonist treatment.

2.6. Confocal immunofluorescence microscopy

Cells growing on poly-D-lysine coated #1 glass coverslips were treated with 5 μM isoproterenol for 18 h or 15 min, washed three times at room temperature with DMEM-H containing nonradioactive CGP-12177 (10 nM), then incubated in complete medium with 10 nM CGP-12177 at 37°C for 60 min. The cells were then washed in PBS containing 0.12% sucrose (PBSS) and fixed with 4% paraformaldehyde in PBSS at 4°C for 10 min, followed by a wash in PBSS. All subsequent steps were performed at room temperature. The fixed cells were incubated in 0.34% L-lysine, 0.05% Na-m-periodate in PBSS for 20 min, then washed in PBSS and permeabilized with 0.2% Triton X-100. After further PBSS wash, the cells were blocked with 10% heat-inactivated goat serum for 15 min. The anti-haemagglutinin epitope monoclonal antibody mHA.11 was diluted to 5 μg/ml in PBSS with 0.2% heat-inactivated goat serum and 0.05% Triton X-100, then added to the cells and left overnight. The cells were washed with PBSS four times before labeling with FITC-anti-mouse IgG (1:100 dilution) using the same procedure as for the

primary antibody. Cover slips were mounted on glass slides using Mowiol (Calbiochem, La Jolla, CA) and viewed with a Molecular Dynamics Multiprobe 2001 confocal imaging system attached to a Zeiss Axiovert 100 microscope. The images were processed using Adobe Photoshop (v. 4.0), and printed on a Codonics NP-1600 dye diffusion printer.

2.7. Transferrin endocytosis

12β6 cells growing in 24-well poly-D-lysine-coated clusters were treated with isoproterenol for 18 h or 15 min, then washed once with DMEM-H. ^{125}I-diferric transferrin in DMEM-H was added to a concentration of 2 nM, then the cells were incubated for varying times at 37°C, chilled and washed once with ice-cold DMEM-H. The monolayers were washed once with ice-cold 150 mM NaCl, 200 mM acetic acid, pH 2.7, and the wash (surface-bound ^{125}I-diferric transferrin), saved for γ-counting. The cellular counts remaining after the acid wash were taken to be the internalized ^{125}I-diferric transferrin, with a correction applied for the acid wash efficiency (58%). The efficiency was determined by binding ^{125}I-diferric transferrin to 12β6 cells at 0°C, applying the acid wash procedure as described above, and comparing the ^{125}I bound before and after acid-wash. The ratio of intracellular to surface ^{125}I-transferrin (In/Sur) was plotted for each time point up to 5 min, and the slope calculated by linear regression to estimate the transferrin endocytic rate constant (Wiley and Cunningham, 1981).

2.8. Transferrin recycling

12β6 cells were incubated with 2 nM ^{125}I-diferric transferrin for 60 min at 37°C to label the total steady-state pool of transferrin receptors. Treatment with isoproterenol was for 17 h prior to and during this labeling period, or during the last 15 min. The cells were washed twice with ice-cold DMEM-H, then brought to 37°C by the addition of warm DMEM-H containing 300 nM unlabeled diferric transferrin and 5 μM isoproterenol. At various times thereafter, the medium was removed and the cells lysed in 0.1% sodium dodecyl sulfate for γ-counting. The fractions of ^{125}I left cell-associated were plotted as a function of time after addition of unlabeled transferrin. Upon reaching endosomes, iron is released from ^{125}I-diferric transferrin, ^{125}I-apotransferrin recycles to the cell surface and is released into the medium. ^{125}I-diferric transferrin remaining on the surface is quite rapidly internalized ($t_{1/2} \sim 3$ min), while release of ^{125}I-apotransferrin due to recycling is much slower ($t_{1/2} \sim 15$ min). Thus, the assay measures the rate-limiting step in the release of cellular ^{125}I-apotransferrin.

3. Results

3.1. Effect of chronic agonist on receptor recycling kinetics

Prolonged agonist exposure might be expected to cause a reduction in the receptor recycling rate constant, thus allowing intracellular receptor concentration to increase and perhaps favoring the occurrence of downregulation. To measure the first-order receptor recycling rate constant (k_r), HEK293 cells expressing haemagglutinin-β_2-adrenoceptors (12β6 cells) were treated for 15 min or 18 h with the full β-adrenoceptor agonist isoproterenol, then washed and suspended in warm medium in the presence of radiolabeled antagonist ([^{3}H]CGP-12177) to allow recycling. In the absence of agonist and in the presence of the antagonist, recycled receptors remain at the cell surface, so that recycling is measured without the complication of concurrent endocytosis (Hertel and Staehelin, 1983; Morrison et al., 1996). The fraction of receptors remaining intracellular were plotted as a function of time after washing away agonist (Fig. 1), and the curves fitted to Eq. (2) (Materials and methods) to derive an estimate of the first-order recycling rate constant, k_r. Internalized receptors in cells exposed to 15 min of agonist recycled with a rate constant k_r of 0.12 ± 0.02 min^{-1}, but after 18 h of agonist, the rate constant was reduced to 0.05 ± 0.02 min^{-1}. These rate constants are significantly different ($P = 0.03$, Student's *t*-test) and correspond to intracellular transit times ($1/k_r$) of 8.3 min and 20.0 min, respectively.

3.2. Downregulation and retention of receptors

The curves in Fig. 1 plateau at surface receptor levels suggesting that after 18 h of agonist exposure, 40–50% of

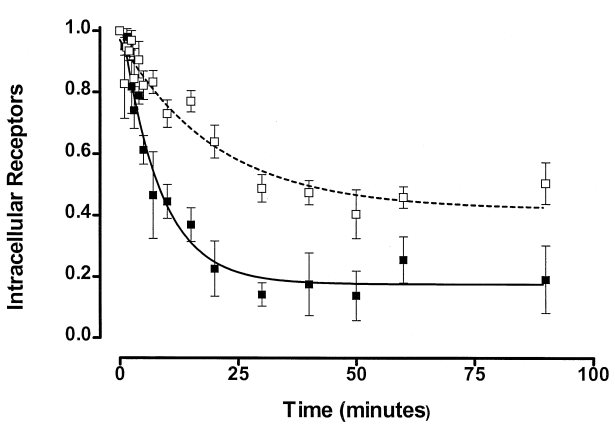


Fig. 1. Receptor recycling after brief or prolonged exposure to agonist. 12β6 cells were treated with 5 μM ISO for 15 min or 18 h, then washed and suspended in warm medium to measure the recycling rate constant as described in Section 2. The first-order rate constants for recycling were derived from individual experiments by curve fitting with Eq. (2) (Section 2), then averaged (see text and Table 1). (■) 15 min of agonist exposure ($N = 4$); (□), 18 h of agonist exposure ($N = 5$).

internalized receptors do not return to the cell surface. Prolonged agonist exposure should cause receptor downregulation, thus we expected the nonrecycling fraction of receptors to be equivalent to the fraction of receptors downregulated. To examine this more closely, 12β6 cells were treated with agonist for varying times, then incubated with [^{3}H]CGP12177 in the presence of digitonin to permit the measurement of total receptor levels. Digitonin treatment permits access of radioligand to internal cellular compartments, as there was no loss of radioligand binding after 15 min of agonist exposure (Fig. 2, grey bars), when downregulation is negligible (Moore et al., 1995) but a large fraction of receptors have internalized (Fig. 2, open bars). After 18 h of agonist exposure, 75 ± 7% of total radioligand binding activity remained compared with no agonist treatment (Fig. 2, grey bars). A similar extent of receptors loss was obtained when measurements were done using cell homogenates and a hydrophobic radioligand ([^{3}H]dihydroalprenolol; data not shown). When agonist was washed away and receptors allowed to recycle for 60 min, only 53% of the receptors were subsequently detected on the surface (Fig. 2, hatched bars). This difference (75% vs. 53%) is significant ($P = 0.03$) and suggests the existence of a pool of receptors that are not downregulated (as defined by radioligand binding activity), but are retained inside cells or recycle with very slow kinetics. Prolonged agonist exposure was required for this to occur, because after 15 min of agonist treatment, all receptors were recovered at the cell surface after 60 min of recycling (117% of control vs. 53% of control, $P = 0.0001$).

Table 1
Receptor levels and rate constants

Time of agonist	R_S^a	R_R^b	R_{SC}^c	R_{IR}^d	k_r (min^{-1})^e	k_e (min^{-1})^f
15 min	0.38	1.17	0.32	0.68	0.12	0.25
18 h	0.22	0.53	0.43	0.57	0.05	0.07

[a] R_S, surface receptors: (surface agonist-treated)/(surface control) (measured in Fig. 2).
[b] R_R, recycled receptors: fraction of total receptors that recycle by 60 min after agonist removal (measured in Fig. 2).
[c] R_{SC}, surface receptors, corrected fraction (R_S / R_R).
[d] R_{IR}, internal recycling receptors ($1 - R_{SC}$).
[e] k_r, measured recycling rate constants, from curve-fitting (Fig. 1).
[f] k_e, calculated endocytic rate constant, from $k_e = k_r(R_{IR})/(R_{SC})$.

3.3. Calculation of endocytic rate constants

The reduction in recycling rate constant could cause an increase in the intracellular fraction of receptors as long as the rate of endocytosis was relatively unaffected. To estimate the first-order rate constant for endocytosis (k_e) after varying times of agonist exposure (Table 1), we used the measured surface levels of receptors (R_S) and the fraction of receptors that recycled within 60 min after agonist removal (R_R). R_S was divided by R_R to yield a corrected term that represents the fraction of the total receptor population remaining on the cell surface after agonist exposure (R_{SC}). After 15 min of agonist treatment, R_S was 0.378, R_R was 1.17, and dividing R_S by R_R gives a value of 0.32 for R_{SC}. The internal fraction of receptors that could recycle (R_{IR}) was then calculated to be 0.68

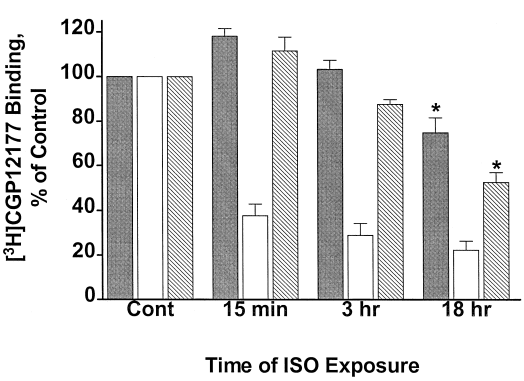


Fig. 2. Total receptor levels and recyclable receptors after agonist exposure. 12β6 cells were cultured in 24-well clusters until ~50% confluent, then left untreated or treated with 5 μM ISO for 15 min, 3 h or 18 h. Total receptors were quantified by radioligand binding with [^{3}H]CGP-12177 in the presence of digitonin (closed bars), while surface receptors were measured in the absence of digitonin (open bars). To ascertain the fraction of recyclable receptors, agonist-treated cells were washed and further incubated with complete medium containing [^{3}H]CGP12177 for 60 min (hatched bars) ($N = 4$). The difference between total radioligand binding fraction left and fraction of recycling receptors is significant, by Student's t-test ($* P = 0.03$).

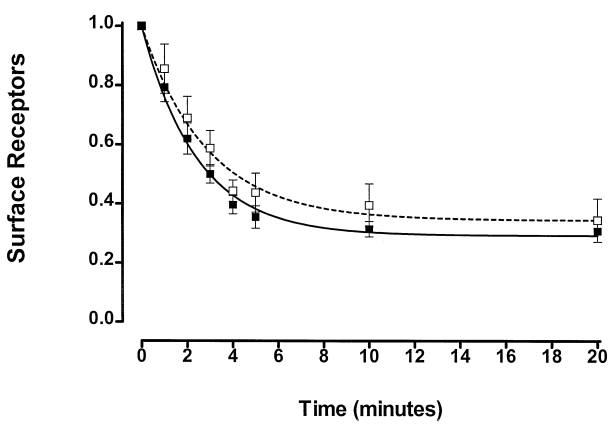


Fig. 3. Internalization of β_2-adrenoceptors after isoproterenol exposure and 60 min of recycling. 12β6 cells growing in 24-well clusters were exposed to 5 μM isoproterenol for 15 min or 18 h, then washed and incubated for 60 min at 37°C to allow recycling to go to completion. The cells were then exposed to 5 μM isoproterenol for varying times, chilled and assayed for surface receptors with [^{3}H]CGP-12177 as described in Section 2. The internalization curves were modeled using Eq. (3) (Section 2). $k_e = 0.29 \pm 0.02$, 15 min; $k_e = 0.23 \pm 0.03$, 18 h ($P = 0.184$, not significant by Student's t-test). $k_r = 0.12 \pm 0.01$, 15 min; $k_r = 0.12 \pm 0.03$ ($P = 0.99$, difference not significant by Student's t-test). $N = 3$ (■), 15 min isoproterenol pre-exposure; (□), 18 h isoproterenol pre-exposure.

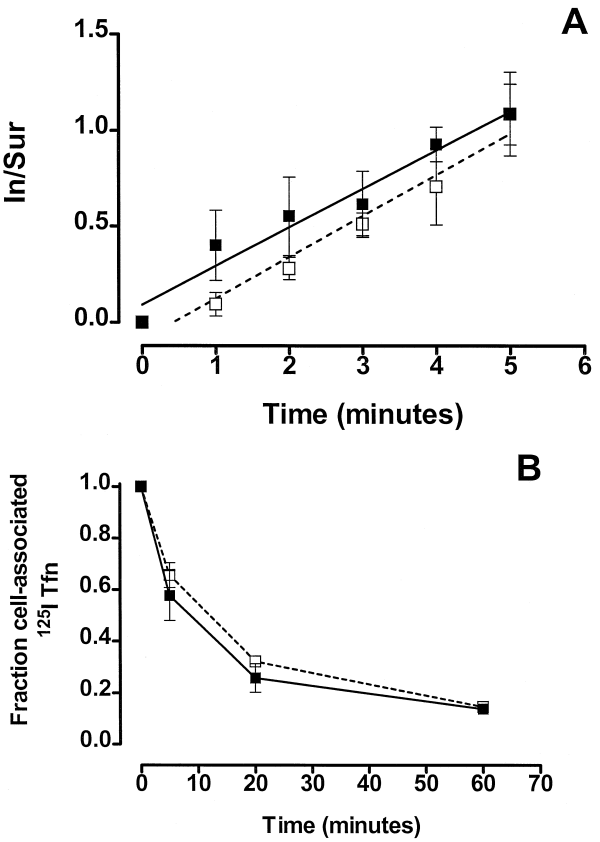


Fig. 4. Effect of agonist on endocytosis and recycling of transferrin receptors. (A). Transferrin endocytosis. 12β6 cells growing in 24-wells clusters were treated with 5 μM isoproterenol for 18 h or 15 min, then incubated with ^{125}I-transferrin for varying times. Internal and surface ^{125}I was determined as described in Section 2. The ratio of surface to intracellular ^{125}I-transferrin (In/Sur) was plotted for each time point up to 5 min, and the slope calculated to provide an estimate of the endocytic rate constant (Wiley and Cunningham, 1981). $k_e = 0.20 \pm 0.03$, 15 min; $k_e = 0.21 \pm 0.03$, 18 h ($P = 0.78$, difference not significant by Student's *t*-test) ($N = 3$). (■), 15 min isoproterenol pre-exposure; (□), 18 h isoproterenol pre-exposure. (B). Transferrin recycling. 12β6 cells growing in 24-well clusters were treated with 5 μM isoproterenol for 18 h or 15 min. The total pool of transferrin receptors was labeled with ^{125}I-transferrin, then the cells were washed and incubated at 37°C in the presence of excess unlabeled transferrin for various times. The fraction of ^{125}I remaining cell-associated was plotted vs. time. (■), 15 min isoproterenol pre-exposure; (□), 18 h isoproterenol pre-exposure.

(1–0.32). Since at steady-state, $k_e = k_r$ (R_{IR}/R_{SC}), a measured recycling rate constant of 0.12 min^{-1} yields a calculated endocytic rate constant of 0.25 min^{-1}. This rate constant is quite similar to that measured previously in this cell line during a 15 min agonist treatment (Morrison et al., 1996). k_e was calculated in a similar way for cells treated with agonist for 18 h, and was found to be 0.07 min^{-1}. Thus, the reduction in the recycling rate constant also was accompanied by a reduction in the endocytic rate constant.

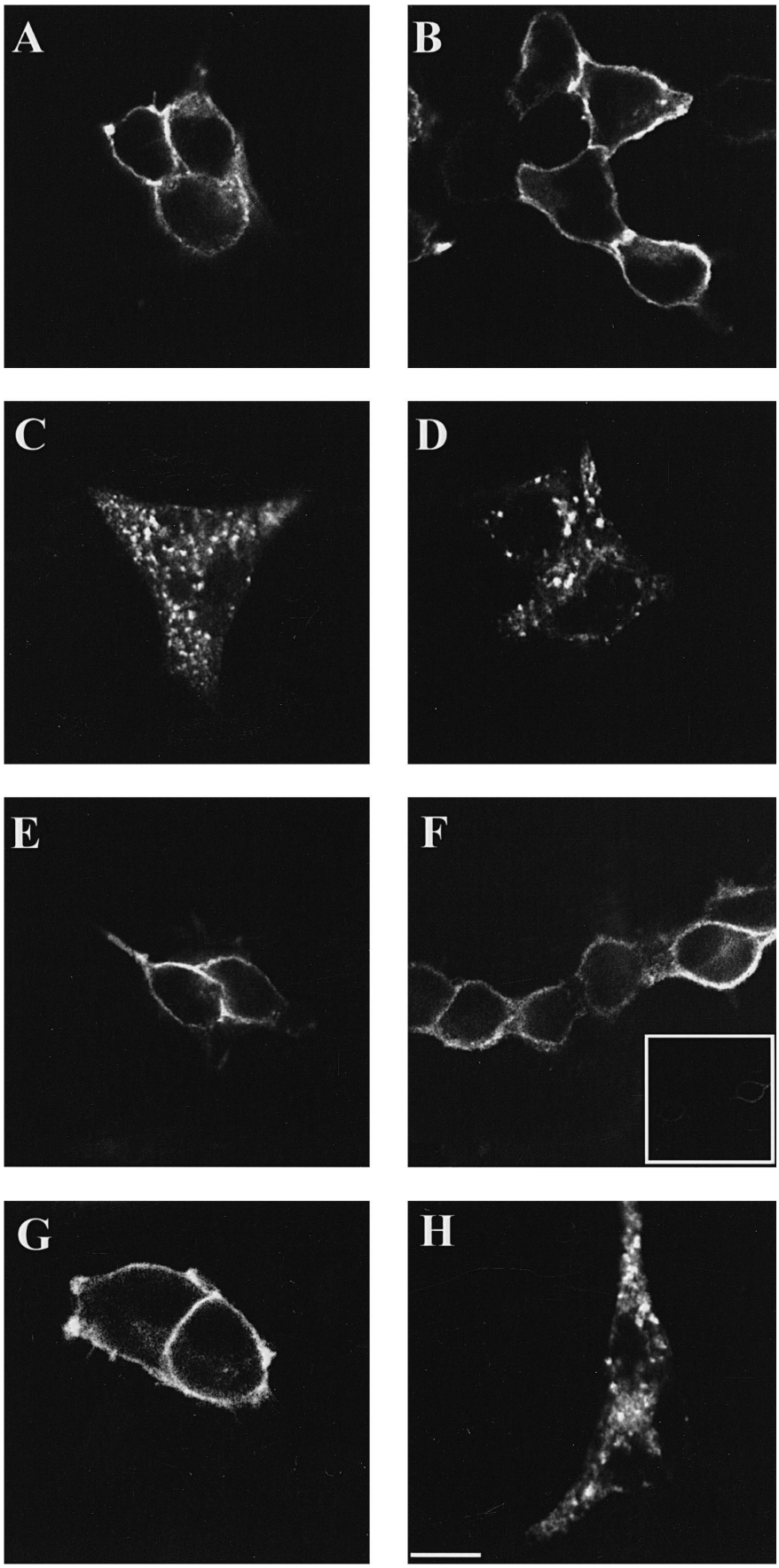
3.4. Reversibility of changes in β_2-adrenoceptor trafficking kinetics

These changes in trafficking kinetics could be caused by nonspecific effects, such as damage to endosomes or other cellular components of the endocytic apparatus. To determine if the effects of prolonged agonist exposure on β_2-adrenoceptor trafficking kinetics were reversible, 12β6 cells were treated with isoproterenol for 15 min or 18 h, washed and incubated at 37°C for 60 min to allow recycling to proceed to completion. These cells were then treated again with isoproterenol and an internalization time course performed. The rate at which surface receptor levels approach a new steady-state after the addition of agonist is a function of the endocytic and recycling rate constants, and the curves can be fitted to obtain unique values for each (Morrison et al., 1996). The effects of prolonged agonist exposure on endocytosis and recycling were fully reversed by 60 min after removal of agonist, since there was no apparent difference in either rate constant after the completion of recycling, regardless of the time of agonist treatment (Fig. 3).

3.5. Specificity of agonist-induced changes in trafficking kinetics

It seemed possible that during prolonged β-adrenoceptor agonist exposure, the kinetics of transfer into and out of endosomes might be altered, thus changing the kinetics of endocytosis and recycling of other receptors. To determine the specificity of prolonged agonist treatment on β_2-adrenoceptor trafficking, we examined the endocytosis and recycling of transferrin receptors, which are known to transit through similar endocytic compartments as internalized β_2-adrenoceptors (Von Zastrow and Kobilka, 1992; Moore et al., 1995). The binding and uptake of ^{125}I-diferric transferrin was measured as a function of time after ^{125}I-diferric transferrin addition, and the endocytic rate constant

Fig. 5. Intracellular β_2-adrenoceptors following isoproterenol treatment and 60 min of recycling. 12β6 cells grown on glass cover slips were exposed to 5 μM isoproterenol for 15 min or 18 h with or without the protease inhibitor leupeptin (100 μM), then fixed, permeabilized and labeled with a monoclonal antibody against the haemagglutinin-tag (mHA.11) as described in Section 2. In some panels, the cells were washed and incubated for 60 min at 37°C in complete medium containing 10 nM CGP-12177 with or without leupeptin before fixation. (A), untreated cells; (B), incubation in the presence of leupeptin for 18 h; (C), incubation with isoproterenol for 15 min; (D), incubation with isoproterenol for 18 h; (E), incubation with isoproterenol for 15 min, then 60 min of recycling; (F), incubation with isoproterenol for 18 h, then 60 min of recycling; (G), incubation with isoproterenol for 15 min, then 60 min of recycling, both in the presence of leupeptin; (H), incubation with isoproterenol for 18 h, then 60 min of recycling, both in the presence of leupeptin. In panel F, the imaging was done at a higher gain compared with panel E to visualize surface receptors. The inset in panel F shows panel F at the same gain as in panel E. Panel H was imaged at a lower gain than panel G to avoid oversaturation by intravesicular receptors. Cells treated as in panel G have surface β_2-adrenoceptors, as measured by [^{3}H]CGP12177 binding (Fig. 6). Scale bar = 10 μm.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was determined according to a previously published method (Wiley and Cunningham, 1981). A plot of the ratio of intracellular ^{125}I to surface ^{125}I (In/Sur) as a function of time is linear over the first 5 min of transferrin uptake, and the slope represents the endocytic rate constant, k_e. There was no significant change in this rate constant for transferrin endocytosis, comparing cells treated with isoproterenol for 15 min with cells treated for 18 h (Fig. 4A). The measured rate constants for transferrin endocytosis (0.20 ± 0.03 min^{-1} for cells treated with agonist for 18 h vs. 0.21 ± 0.03 min^{-1} for cells treated with agonist for 15 min) are quite similar to rates reported in other cell types (Schonhorn and Wessling-Resnick, 1994). To measure the recycling of transferrin, 12β6 cells were incubated with ^{125}I-diferric transferrin for 60 min to equilibrate the intracellular pool of transferrin receptors. Intracellular ^{125}I-diferric transferrin bound to transferrin receptors releases iron in the low pH environment of endosomes, remains bound to receptors as ^{125}I-apotransferrin, and is released into the medium upon return of receptors to the cell surface and exposure to neutral pH (Klausner et al., 1983). The labeled cells were washed and incubated at 37°C to allow recycling, and excess unlabeled diferric transferrin was included in the incubation medium to prevent binding of released ^{125}I-apotransferrin. A plot of the fraction of ^{125}I remaining cell-associated vs. time (Fig. 4B) is similar after 15 min or 18 h of exposure to isoproterenol, suggesting that there was no significant effect of prolonged isoproterenol treatment on transferrin receptor recycling.

3.6. Intracellular sorting of β_2-adrenoceptors during chronic agonist treatment

We next determined whether prolonged agonist exposure caused changes in intracellular receptor sorting that might be related to measured reductions in receptor trafficking kinetics and intracellular retention of receptors. Agonist-treated 12β6 cells were fixed, permeabilized and labeled with mHA.11, a monoclonal antibody against the haemagglutinin-tag on the N-terminus of the β_2-adrenoceptor. After 15 min or 18 h of agonist treatment, there was a robust appearance of receptors within intracellular vesicles (Fig. 5C,D), compared with cells not exposed to agonist (Fig. 5A,B). When the cells were washed and then incubated at 37°C for 60 min prior to labeling with antibody, internalized receptors recycled to the cell surface (Fig. 5E,F). The overall fluorescent signal was very weak in cells exposed to 18 h of agonist (Fig. 5F, inset), and could be seen only if the gain was increased in the confocal imaging system (Fig. 5F). In contrast, when the protease inhibitor leupeptin was included in the medium during the 18 h agonist exposure and subsequent recycling, a considerable fraction of receptor was left behind in intracellular vesicles (Fig. 5H). This finding suggests that receptors had entered a protease-containing compartment where, in the absence of leupeptin, the haemagglutinin-tag normally would be degraded. Intracellular retention of receptors was not caused by the inhibition of recycling by leupeptin, because the drug had no significant effect on recycling in cells exposed to agonist for only 15 min (Fig. 5G). Exposure to leupeptin itself for 18 h did not cause the intracellular accumulation of receptors (Fig. 5B), and had little or no effect on the kinetics of β_2-adrenoceptor endocytosis and recycling, or on the endosomal distribution of transferrin receptors (data not shown).

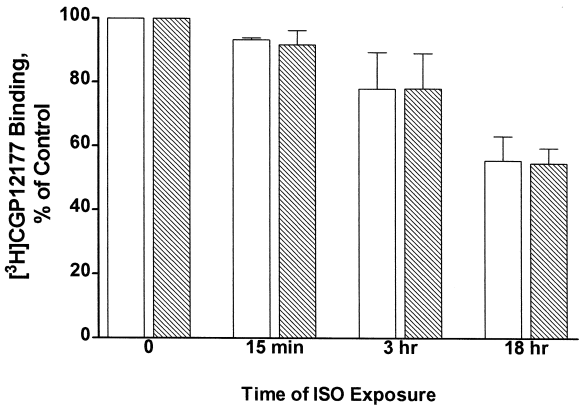

Fig. 6. Receptor recycling in the presence of leupeptin. 12β6 cells were cultured in 24-well clusters until ~50% confluent, then left untreated or treated with 5 μM isoproterenol for 15 min, 3 h or 18 h in the absence (open bars) or presence (hatched bars) of 100 μM leupeptin. The cells were then washed and further incubated at 37°C with complete medium containing 6 nM [^{3}H]CGP12177 for 60 min to allow recycling. The clusters were then incubated at 4°C for 60 min prior washing and quantification of bound label as described in Section 2 ($N = 3$).

3.7. Effect of protease inhibitor on the fraction of nonrecycling receptors

We next asked whether proteolytic action on receptors per se was sufficient to prevent them from recycling. Because intracellular haemagglutinin-tagged β_2-adrenoceptors cannot be detected after 18 h of agonist exposure unless leupeptin is included in the medium, we infer that receptors traffic through a compartment containing proteases that degrade the epitope tag (Fig. 5). Thus, we treated 12β6 cells with agonist for varying times in the presence or absence of leupeptin, then washed the cells and incubated them for 60 min to permit recycling. If protease action were sufficient to prevent the recycling of receptors after 18 h of agonist treatment, then in the presence of leupeptin more receptors should recycle. In fact, the presence of leupeptin during the 18 h agonist treatment had no effect on the fraction of receptors that recycled by 60 min after removal of agonist (Fig. 6).

4. Discussion

This study was undertaken to discover changes in receptor trafficking or sorting events associated with β_2-adren-

oceptor downregulation during prolonged agonist exposure. First, we found that the first-order rate constant for β_2-adrenoceptor recycling from intracellular compartments to the plasma membrane was reduced, from 0.12 min^{-1} to 0.05 min^{-1}. This finding is significant because the reduced recycling rate constant reflects an increased intracellular transit time $(1/k_r)$ for receptors, from 8.3 min after a brief agonist treatment, to 20.0 min after 18 h of agonist. The extended transit time could play a role in downregulation by increasing the duration of exposure of intracellular receptors to diversion into lysosomes for degradation. Alternatively, the increased transit time could reflect the occurrence of additional steps, such as vesicular transport events, in the process leading from intracellular compartments to the plasma membrane. If such additional steps were occurring, the 20.0 min transit time would reflect a composite of several different transit times.

Another significant event occurring with prolonged agonist exposure is the diversion of ligand-binding receptors into an intracellular compartment from which recycling is very slow or nonexistent. Our data suggest that these nonrecycling receptors are exposed to proteases, since their haemagglutinin-tags are observed by immunofluorescence microscopy only in the presence of the protease inhibitor leupeptin (Fig. 5H). However, these nonrecycling receptors do not appear to be downregulated: after 18 h of agonist exposure, loss of radioligand binding sites amounted to about 25%, while the loss of receptors as measured by recycling in the absence of protease inhibitor was 47% (Fig. 2). Moreover, the presence of protease inhibitor did not change the fraction of receptors that failed to recycle (Fig. 6), suggesting that the activity of proteases per se was not sufficient to inactivate receptor recycling capacity. The simplest interpretation of these findings is that once receptors enter this protease-containing compartment, there is an irreversible commitment to downregulation that occurs prior to the loss of radioligand binding activity.

The identity of this compartment cannot be definitely ascertained from current information, however a likely possibility would be a prelysosomal compartment such as the late endosome. This compartment becomes enriched for endocytic tracers by about 10 min after addition to cells, as distinct from early endosomes, where tracers are found within 3–5 min (Schmid et al., 1988). Late endosomes also are distinctively enriched in the ras-related GTPase rab7 (Méresse et al., 1995) and in cation-independent mannose-6-phosphate receptors, which are required for shuttling lysosomal proteases from the *trans*-Golgi network (Griffiths et al., 1988). Ligands internalized by receptor-mediated endocytosis (Gruenberg and Maxfield, 1995) and certain membrane proteins (Feng et al., 1995) are capable of trafficking to late endosomes from early endosomes, possibly by way of specific carrier vesicles (Aniento and Gruenberg, 1995). Late endosomes also are sites where newly synthesized proteases undergo proteolytic processing (Delbrück et al., 1994), thus haemagglutinin-β_2-adrenoceptors entering such vesicles would likely undergo some degree of degradation and lose their epitope tags. However, removal of only the β_2-adrenoceptor N-terminal domain would probably not reduce ligand-binding, since this function is unaffected by deletion of the receptor N-terminus (Dixon et al., 1987).

Measurements of the receptor levels in these cellular compartments, together with determinations of recycling rate constants, permitted a calculation of the receptor endocytic rate constants after brief or prolonged agonist exposure. To perform this analysis, the total receptor levels were adjusted to reflect the fact that 47% were essentially nonrecyclable after 18 h of agonist exposure. Because the relative fractions of surface and recycling internal receptor levels did not differ greatly when comparing 15 min of agonist with 18 h, the endocytic rate constant (as calculated) must have been considerably reduced. Thus, while a low rate of receptor recycling would ordinarily serve to increase the intracellular concentration of receptors available for downregulation, this effect appears to be counteracted after prolonged agonist exposure by a reduced rate of endocytosis. There are in fact many other circumstances where β_2-adrenoceptor downregulation occurs even with reduced rates of endocytosis. Certain mutant β_2-adrenoceptors are defective in rapid endocytosis yet still undergo agonist-induced downregulation (Hausdorff et al., 1991; Barak et al., 1994). Also, partial β-adrenoceptor-agonists cause substantial downregulation of β_2-adrenoceptors (albeit at somewhat slower rates) (Pittman et al., 1984), despite greatly reduced rates of receptor endocytosis (Morrison et al., 1996; January et al., 1997). These previous findings, together with the results presented here, suggest that downregulation is an efficient process requiring only some degree of receptor endocytosis to occur, and does not necessarily require the increased concentration of receptors within endosomes. Instead, our data suggest that downregulation is dependent on slow intracellular transit times and specific intracellular sorting events.

Several mechanisms to explain reduced β_2-adrenoceptor trafficking rates can be ruled out. First, the reversibility of the effect of prolonged agonist on the rates of β_2-adrenoceptor endocytosis and recycling suggests that there is no loss of cell viability, nor does there appear to be permanent damage to the endocytic or recycling machinery (Fig. 3). Secondly, the endocytosis and recycling of transferrin receptors are indistinguishable in cells exposed to agonist for 15 min or 18 h (Fig. 4), suggesting that there are no acute effects on the constitutive endocytic apparatus. Thus, our data indicate that the agonist-induced changes in the β_2-adrenoceptor trafficking kinetics are both specific and reversible.

In conclusion, the present studies provide evidence for specific changes in β_2-adrenoceptor trafficking events during downregulation. Reductions in the first-order rate constants for both endocytosis and recycling after prolonged agonist treatment are accompanied by changes in the intra-

cellular localization of receptors to a vesicular compartment that appears to contain proteases. Since movement out of this compartment is very slow or nonexistent, it probably represents an intermediate between endosomes and lysosomes on the downregulation pathway. Additional work is needed to define this putative intermediate and to discover the mechanism of reduced receptor trafficking kinetics during prolonged agonist exposure.

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