

Antagonist-dependent and -independent Steps in the Mechanism of Adrenergic Receptor Internalization*

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Epitope tagging and immunocytochemical techniques were used to examine the agonist-regulated internalization of human β_2 -adrenergic receptors in 293 cells. In the absence of agonist, receptors tagged with monoclonal antibody remain in the plasma membrane for >1 h. In the presence of agonist, tagged receptors are endocytosed within 10 min. Endocytosed receptors are located in endosomes and can be recycled to the plasma membrane. In the prolonged presence of agonist, receptor endocytosis continues even after maximum sequestration of surface receptors (measured by radioligand binding to intact cells) has occurred. The process of receptor endocytosis requires cellular ATP and is temperature-dependent. At 4 °C, no agonist-induced redistribution of receptors located in the plasma membrane is observed. At 16 °C, agonist causes receptors to cluster in and around coated invaginations of the plasma membrane, but receptor endocytosis does not occur. Agonist treatment of cells at 16 °C, but not 4 °C, predisposes receptors to agonist-independent endocytosis upon warming to 37 °C. These studies suggest that: 1) β_2 -adrenergic receptors reside stably in the plasma membrane of untreated cells, while they continuously cycle between the plasma membrane and endosomes in the presence of agonist; 2) agonist regulates an early step in the endocytosis mechanism, which is associated with the redistribution of adrenergic receptors between distinct microdomains of the plasma membrane; and 3) later steps in the endocytosis mechanism do not require agonist and may utilize the same endocytic machinery that mediates the endocytosis of constitutively recycling receptors.

The physiological regulation of G protein-coupled receptors is determined by multiple processes. Adrenergic receptors undergo phosphorylation, down-regulation, and sequestration in response to stimulation by agonist. Receptor phosphorylation and down-regulation have been studied in detail. These processes are thought to function, respectively, in short and long term desensitization of receptor signaling (1–5). Receptor sequestration is not well understood. It has been suggested that sequestration plays a role in resensitizing β_2 -adrenergic receptors following agonist-induced phosphorylation (6, 7), and a similar role has been suggested in studies of α -mating factor receptor in yeast (8). However, the mechanisms that mediate receptor sequestration and resensitization are not understood.

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Studies using ligand binding and subcellular fractionation techniques suggest that sequestration involves the internalization of receptors from the plasma membrane (4, 9, 10). Immunocytochemical studies utilizing receptor-specific antibodies confirm this hypothesis (11, 12). In 293 cells, internalized β_2 -receptors accumulate in the same endosomes that contain constitutively recycling transferrin receptors (12). These findings raise two related questions. First, can adrenergic receptors be internalized by the same mechanism that mediates constitutive endocytosis? Second, if this is so, how is the entry of adrenergic receptors into this mechanism regulated by agonist?

We previously applied epitope tagging, a general method for protein localization and purification (13, 14), to the localization of G protein-coupled receptors in fixed specimens (12, 15). We have now extended this method to monitor the movement of β_2 -adrenergic receptors in living cells, to examine the distribution of receptors in the plasma membrane at high resolution, and to begin to dissect the mechanism by which agonist regulates receptor internalization.

EXPERIMENTAL PROCEDURES

Cell Culture and Radioligand Binding—Wild type human β_2 -adrenergic receptor and a mutant receptor possessing an amino-terminal epitope tag recognized by 12CA5 monoclonal antibody were expressed in stably transformed clones of 293 human kidney fibroblast cells (ATCC) as described elsewhere (12). Cell lines studied expressed between 400 and 900 fmol of receptor/mg of protein as determined by [³H]dihydroalprenolol binding assay in a crude cell membrane preparation. Sequestration assays were performed by incubating intact cells with 10 nM ³H-labeled CGP12177 (Amersham Corp.), using 1 μ M alprenolol to define nonspecific binding, as previously described (12).

Trafficking of Receptors Tagged in the Plasma Membrane with Monoclonal Antibody—Glass coverslips containing 293 cells expressing epitope-tagged β_2 -receptors were chilled on ice in Dulbecco's minimal essential media (UCSF Cell Culture Facility) supplemented with 10% fetal bovine serum (HyClone) and 30 mM sodium HEPES, pH 7.4. After chilling for 10 min, 12CA5 monoclonal antibody (BAbCO) was added (5 μ g/ml), and cells were incubated on ice for 60 min. Unbound antibody was removed by washing with several changes of HEPES-buffered medium at 4 °C, then cells were warmed to 37 °C. After the indicated treatments, cells were fixed in PBS¹ containing 4% formaldehyde and permeabilized with 0.2% Nonidet P-40 (Sigma). Fixed, permeabilized specimens were then incubated with a rabbit antiserum recognizing the carboxyl terminus of the β_2 -receptor (12). Both antibodies (12CA5 monoclonal antibody labeling receptors located initially in the plasma membrane, and antiserum labeling the full complement of cellular β -receptors) were detected in the same specimens using species-specific antibodies labeled with fluorochromes (FITC-goat anti-mouse and Texas Red donkey anti-rabbit IgGs, Jackson ImmunoResearch). Double-label immunofluorescence microscopy was performed using a custom-built laser scanning confocal microscope and a Nikon 60X NA1.3 objective (Dr. Stephen Smith, Cell Sciences Imaging Facility, Program in Molecular and Genetic Medicine, Stanford Medical Center). Negligible bleed-through between channels was confirmed using single-labeled specimens.

¹ The abbreviations used are: PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody.

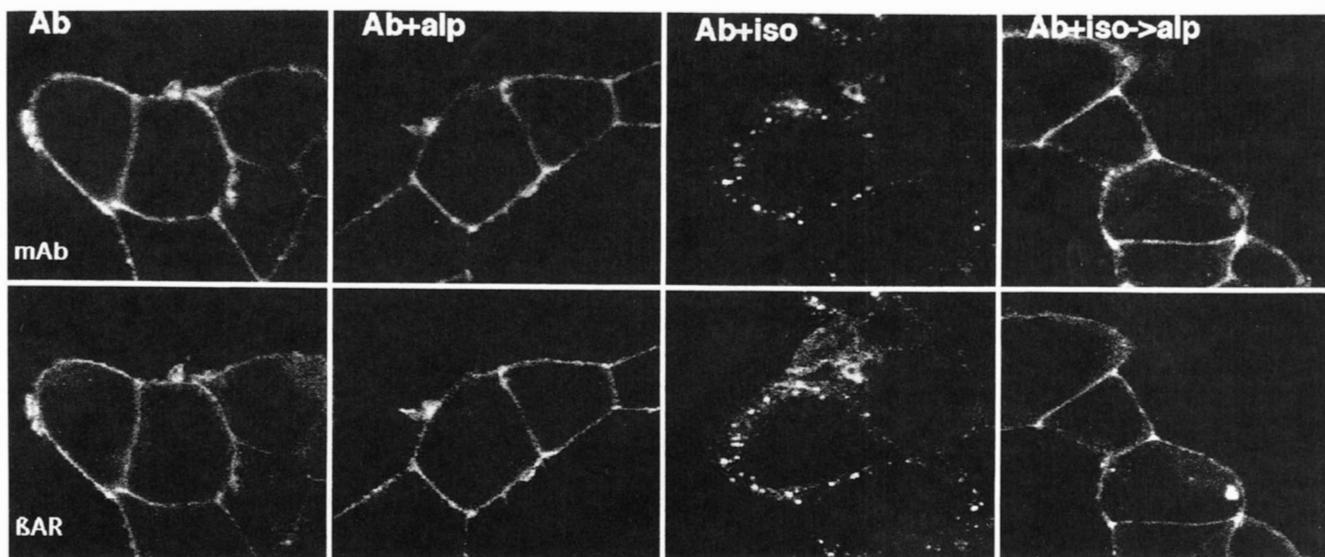


Fig. 1. Endocytosis and recycling of mutant β_2 -adrenergic receptors tagged in the plasma membrane with monoclonal antibody. Receptors present in the plasma membrane of 293 cells stably expressing epitope-tagged β_2 -receptors were selectively labeled by 12CA5 mAb and washed at 4 °C as described under "Experimental Procedures." Labeled cells were equilibrated to 37 °C for 10 min prior to the following treatments. *Ab*, incubation without added drugs for 1 h; *Ab+alp*, incubation for 1 h in the presence of 10 μ M alprenolol; *Ab+iso*, incubation for 50 min in the absence of drugs followed by 10 min in the presence of 10 μ M isoproterenol; *Ab+iso->alp*, incubation for 30 min in the absence of drugs, followed by 10 min with 10 μ M isoproterenol, followed by removing medium and replacing it with medium containing 10 μ M alprenol for an additional 20 min. After these incubations, cells were fixed, permeabilized, and processed for dual immunolocalization of 12CA5 mAb (*top panels*) and β_2 -receptors detected using antiserum recognizing a carboxyl-terminal epitope (*bottom panels*). Confocal fluorescence microscopy was performed through cell monolayers using a plane of focus adjusted approximately 4 μ m above the surface of the coverslip.

Receptor-mediated Endocytosis of Monoclonal Antibody—Cells expressing epitope-tagged receptors were grown on coverslips in Dulbecco's minimal essential medium containing 10% fetal bovine serum and 30 mM HEPES, pH 7.4. 12CA5 monoclonal antibody (5 μ g/ml) was added to the medium, and, after the indicated incubations, medium was removed, monolayers were quickly washed in PBS, and cells were fixed in PBS containing 4% formaldehyde. Secondary staining using FITC-labeled goat anti-mouse IgG and immunofluorescence microscopy were performed as above.

Receptor Localization in the Plasma Membrane—Cells expressing epitope-tagged receptors, grown on glass coverslips, were treated as indicated then rapidly chilled on ice in HEPES-buffered medium. Receptors present in the plasma membrane were labeled by adding 12CA5 antibody to the culture medium and incubating on ice for 60 min prior to fixation in ice-cold PBS containing 4% formaldehyde. Subsequently, antibody-labeled receptors were detected using FITC-goat anti-mouse IgG and immunofluorescence microscopy, using a Zeiss Axiohot microscope and a Zeiss 100X/NA1.3 objective. For immunoelectron microscopy, cells were grown on plastic coverslips (Thermonox). Cells were treated, chilled, and incubated with 12CA5 monoclonal antibody as for immunofluorescence. Subsequently cells were fixed in ice-cold PBS containing 2% freshly dissolved paraformaldehyde, washed at room temperature for 30 min in PBS containing 2% bovine serum albumin and 5% normal goat serum, incubated at room temperature with affinity-purified rabbit anti-mouse IgG (5 μ g/ml, Jackson ImmunoResearch) for 45 min, washed in PBS/bovine serum albumin/goat serum, incubated in 1:100 dilution of protein A-colloidal gold (Amersham Corp.) for 60 min in PBS/bovine serum albumin/goat serum prior to washing and post-fixation with 4% paraformaldehyde and 0.2% glutaraldehyde. Specimens were osmicated, dehydrated, and embedded in Epon. Ultrathin sections were cut, placed on Formvar-coated grids, and examined using a Philips 310 transmission electron microscope at 80 kV. Specificity of gold staining was confirmed by staining untransfected 293 cells, as well as cells expressing β_2 -receptors not containing the epitope tag. To quantitate the distribution of receptors observed by electron microscopy, microscopic fields were selected at random and photographed at 21,000 \times magnification. Gold particles in the micrographs were classified according to the following patterns of localization and counted: 1) microvillar extensions, gold particles associated with 75–300-nm diameter tubular extensions of the plasma membrane; 2) membrane pits, gold particles found in or within 100 nm of 50–150-nm diameter invaginations of the plasma membrane; 3) flat regions, gold particles associated with portions of the plasma membrane greater than 100 nm from either a microvillar extension or

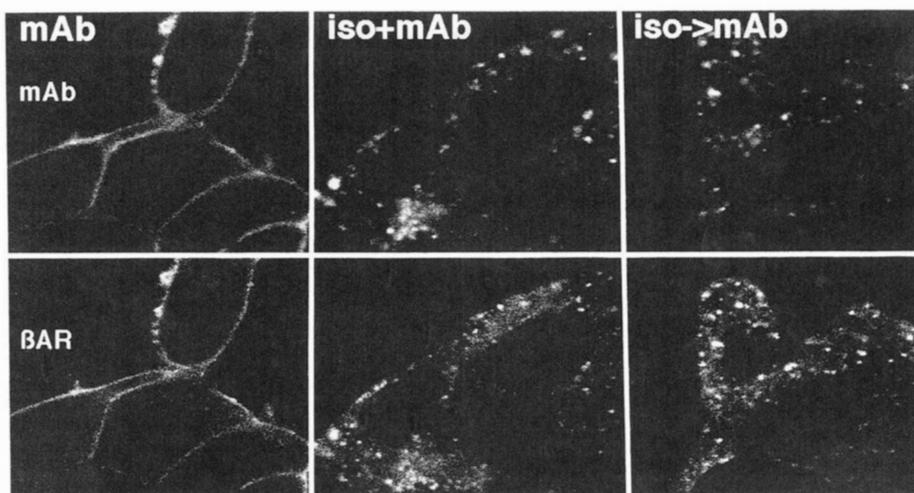
membrane pit; and 4) intracellular, gold particles associated with vesicles or other intracellular structures.

RESULTS AND DISCUSSION

Agonist-regulated Endocytosis of β_2 -Receptors Followed by Recycling to the Plasma Membrane—Receptors present in the plasma membrane of stably transfected 293 cells were tagged by incubating intact cells with monoclonal antibody at 4 °C, then cells were washed to remove excess unbound antibody. 12CA5 antibody was chosen because this antibody binds with extremely high affinity and does not dissociate under harsh conditions (13), therefore it would not be expected to dissociate in the mildly acidic endosomal environment (16). After various treatments at 37 °C, the movement of receptors residing initially in the plasma membrane was determined by localizing tagged receptors in fixed, permeabilized cells using confocal immunofluorescence microscopy (Fig. 1, *upper panels*). No detectable endocytosis of surface-tagged receptors was observed in the absence of agonist (Fig. 1, *Ab*) or presence of the antagonist alprenolol for 1 h (Fig. 1, *Ab+alp*). Agonist (10 μ M isoproterenol) caused receptors tagged in the plasma membrane to be endocytosed within 10 min (Fig. 1, *Ab+iso*). Following treatment of cells with agonist, removal of agonist and addition of antagonist caused endocytosed receptors to recycle back to the plasma membrane (Fig. 1, *Ab+iso->alp*). 12CA5 monoclonal antibody colocalized with receptors detected independently by a receptor-specific antiserum (12), confirming that monoclonal antibody remained associated with receptors throughout a complete round of endocytosis and recycling (Fig. 1, *lower panels*). These results indicate that receptors undergo agonist-regulated endocytosis, and endocytosed receptors can efficiently recycle to the plasma membrane.

Agonist-regulated Internalization Is a Dynamic Process—We tested two hypotheses to explain the agonist-regulated endocytosis and recycling of receptors observed in these experiments. First, agonist-induced internalization could result from a static process in which a proportion of receptors are endocytosed to a storage pool until agonist is removed, and endocytosed recep-

FIG. 2. Continuous receptor-mediated endocytosis of monoclonal antibody in the presence of agonist. 12CA5 mAb added to the culture medium was used to detect endocytosis of epitope-tagged β_2 -receptors, as described under "Experimental Procedures." The following conditions are shown: *mAb*, incubation with 12CA5 antibody for 15 min in the absence of drugs; *iso+mAb*, incubation with 12CA5 antibody and 10 μM isoproterenol for 15 min; *iso-mAb*, incubation with 10 μM isoproterenol in the absence of mAb for 60 min, followed by 15 min with 12CA5 mAb in the continued presence of 10 μM isoproterenol. After these incubations, cells were fixed, permeabilized and processed for dual immunolocalization of mAb (*top panels*) and β_2 receptors detected by carboxyl-terminal antiserum (*bottom panels*). Confocal microscopy was performed as in Fig. 1.



tors return to the plasma membrane. In this case, agonist-induced endocytosis of receptors would be expected to occur transiently and to stop when receptor internalization plateaus at its maximum value (approximately 50%) which occurs within 10–15 min after agonist addition, as determined by sequestration of CGP-12177 binding sites from the surface of intact cells (12, 17). Alternatively, agonist-induced internalization could represent a dynamic process, in which receptors continuously undergo endocytosis and recycling in the presence of agonist. In this case, receptor endocytosis would be expected to occur both immediately upon adding agonist as well as at later times, even after agonist-induced internalization has reached steady state. To test these hypotheses, receptor-mediated uptake of 12CA5 monoclonal antibody from the culture medium was used to assay receptor endocytosis at various times after the addition of agonist. This endocytosis assay was possible because antibody does not induce or block receptor internalization (see above) and antibody uptake was specifically receptor-mediated. No uptake of monoclonal antibody was detected in the absence of receptor internalization (see below), in untransfected cells or in cells expressing β_2 -receptors without the 12CA5 epitope tag (data not shown). As in the first set of experiments, receptors were localized following cell fixation using carboxyl-terminal antiserum, showing that 12CA5 antibody was endocytosed to the same endosomes containing internalized receptors (Fig. 2, *upper and lower panels*). Addition of 12CA5 antibody to the culture medium in the absence of agonist caused labeling of epitope-tagged receptors present in the plasma membrane, but no receptor-mediated endocytosis of 12CA5 antibody was observed (Fig. 2, *mAb*). As predicted by either hypothesis, receptor-mediated uptake of antibody to receptor-containing endosomes was observed immediately after the addition of isoproterenol (Fig. 2, *iso+mAb*). However, receptor-mediated endocytosis of antibody continued to occur in the prolonged presence of agonist. One hour after the addition of isoproterenol, 12CA5 antibody added to the culture medium continued to be rapidly endocytosed in the same manner (Fig. 2, *iso-mAb*). As in the case of shorter agonist treatment, receptors endocytosed after 1 h colocalized with transferrin receptors and returned to the plasma membrane following removal of agonist (not shown). These results are consistent with the hypothesis that agonist-induced internalization of receptors is a dynamic process, in which receptors undergo continuous endocytosis and recycling in the presence of agonist. In addition, these results confirm that agonist regulates the rate of receptor endocytosis.

Agonist Causes a Redistribution of Receptors within the Plasma Membrane That Can Be Dissociated from Receptor En-

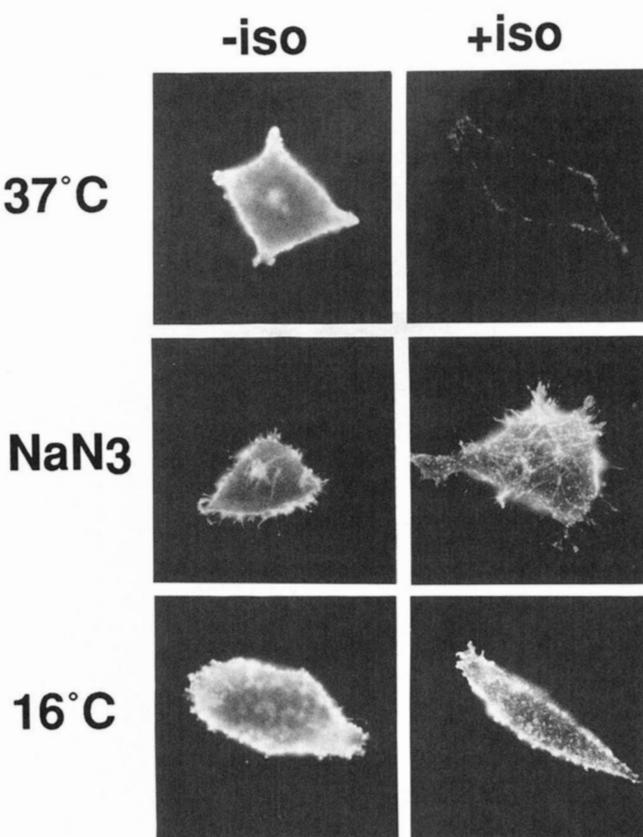


FIG. 3. Inhibition of β_2 -receptor internalization by sodium azide and reduced temperature. 293 cells expressing epitope-tagged β_2 -receptors were subjected to the following treatments prior to chilling cells to 4 °C and selectively immunostaining receptors present in the plasma membrane using 12CA5 mAb in nonpermeabilized cells, as described under "Experimental Procedures." 37 °C (*top panels*), cells were treated for 15 min at 37 °C in the absence (*-iso*) or presence (*+iso*) of 10 μM isoproterenol; NaN_3 (*middle panels*), cells were incubated for 30 min at 37 °C in glucose-free PBS containing 0.04% sodium azide prior to an additional 15-min incubation in the absence (*-iso*) or presence (*+iso*) of 10 μM isoproterenol; 16 °C (*bottom panels*), cells were equilibrated to 16 °C for 30 min in normal growth medium supplemented with 30 mM sodium HEPES, pH 7.4, followed by an additional 15-min incubation at 16 °C in the absence (*-iso*) or presence (*+iso*) of 10 μM isoproterenol. After immunostaining and fixation, surface receptors were visualized by conventional immunofluorescence microscopy, with the focal plane centered approximately 3 μm above the coverslip. Photography was performed using identical exposure times to allow comparison of surface immunofluorescence intensity.

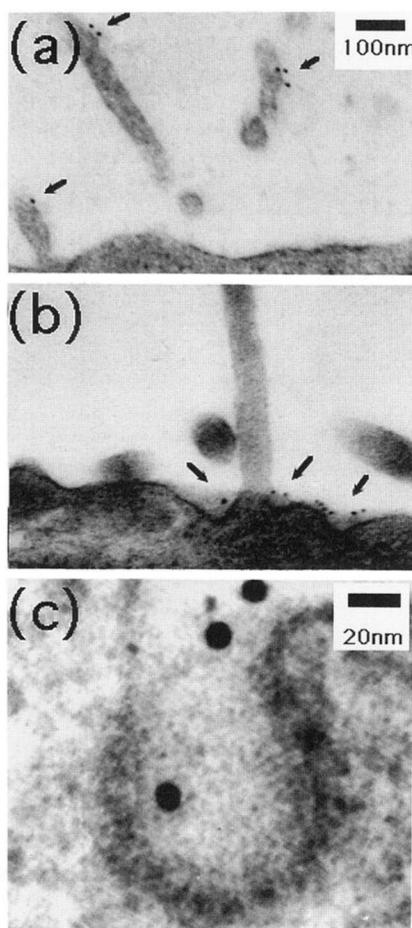


FIG. 4. Immunoelectron microscopy of β_2 -receptors in the plasma membrane. 293 cells expressing epitope-tagged β_2 -receptors were equilibrated to 16 °C and incubated for 15 min in the absence (panel *a*) or presence (panels *b* and *c*) of 10 μ M isoproterenol as in Fig. 3. Following these incubations, cells were rapidly chilled on ice, receptors present in the plasma membrane were labeled with 12CA5 mAb, and specimens were processed for electron microscopy as described under “Experimental Procedures.” Representative fields from transmission electron microscopy are shown, and receptors labeled with gold particles are indicated by arrows. Panels *a* and *b* were photographed at the same magnification, while Panel *c* shows a higher magnification view of a typical receptor-containing invagination observed in the plasma membrane of isoproterenol-treated cells. Receptor redistribution was quantitated by counting gold particles in 35 randomly selected microscopic fields photographed from each specimen at 21,000 \times magnification, as described under “Experimental Procedures.” In untreated cells, 1.3% of gold particles were localized in or within 100 nm of a plasma membrane pit and 47% were associated with microvillar extensions of the plasma membrane ($n = 315$). In isoproterenol-treated cells, 18% of gold particles were localized in or within 100 nm of plasma membrane pits, and 29% were associated with microvillar extensions ($n = 522$). The remainder of gold particles were associated with flat regions of the plasma membrane greater than 100 nm from either a pit or microvillar extension. Less than 1% of gold particles observed in either specimen were associated with intracellular structures.

docytosis—Agonist-regulated receptor endocytosis could be dissected into steps differing in temperature dependence and sensitivity to reduced ATP levels. After various treatments, receptors remaining in the plasma membrane were visualized by immunocytochemical staining of nonpermeabilized cells, after chilling cells to 4 °C (to stop receptor internalization). Treatment of cells with agonist at 37 °C caused a substantial loss of receptors detected in the plasma membrane, consistent with receptor endocytosis (Fig. 3, top panels). Upon closer examination, receptors remaining in the plasma membrane of agonist-treated cells appeared to be localized in a punctate or clustered distribution in the plasma membrane (Fig. 3, top right panel).

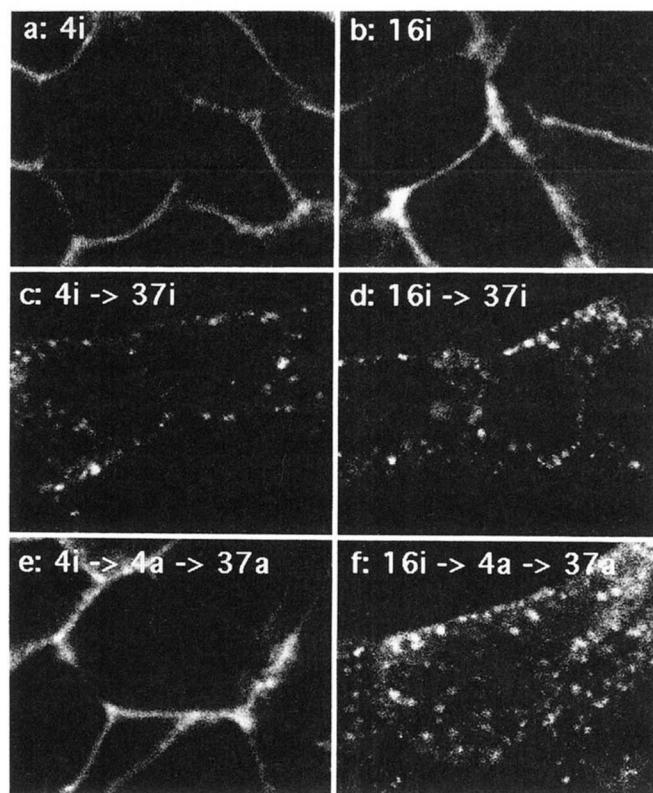


FIG. 5. Agonist exposure at 16 °C predisposes β_2 -receptors to subsequent agonist-independent internalization. 293 cells expressing epitope-tagged β_2 -receptors were treated under the following conditions prior to fixation, permeabilization and localization of receptors using confocal immunofluorescence microscopy, as described under “Experimental Procedures.” *a*, cells were equilibrated at 4 °C for 30 min, then incubated in the presence of 10 μ M isoproterenol for an additional 30 min at 4 °C. *b*, cells were equilibrated at 16 °C and incubated with 10 μ M isoproterenol for 30 min at 16 °C. *c*, cells were equilibrated and incubated at 4 °C with isoproterenol as in (*a*), then warmed to 37 °C for 10 min prior to fixation. *d*, cells were equilibrated and incubated at 16 °C with isoproterenol as in (*b*), then warmed to 37 °C for 10 min prior to fixation. *e*, cells were equilibrated and incubated with isoproterenol at 4 °C as in (*a*), then washed at 4 °C with three changes of media containing 10 μ M alprenolol over 45 min, then warmed to 37 °C for 10 min prior to fixation. *f*, cells were equilibrated and incubated with isoproterenol at 16 °C as in (*b*), then chilled on ice and washed at 4 °C with three changes of media containing 10 μ M alprenolol over 45 min, then warmed to 37 °C for 10 min prior to fixation. Confocal optical sections imaged through the center of a representative cluster of several cells is displayed to allow the differentiation of receptors in the plasma membrane (staining of cell periphery seen in Panels *a*, *b*, and *e*) from receptors internalized to endosomal vesicles (punctate distributed inside the cell margins). Confocal imaging was performed as in Fig. 1. The much finer-grained clustered appearance of receptors in the plasma membrane of cells, observable using conventional fluorescence microscopy, is difficult to observe in these confocal sections.

In cells preincubated with sodium azide in the absence of glucose to reduce intracellular ATP levels (18), no agonist-induced loss of receptors from the plasma membrane was observed. However, agonist caused receptors in ATP-depleted cells to appear clustered in the plasma membrane (Fig. 3, middle panels). Receptor endocytosis was also blocked by reduced temperature. When cells were incubated at 4 °C, no agonist-induced endocytosis or clustering of receptors was observed (not shown). At 16 °C, however, receptor endocytosis was blocked but agonist caused receptors to appear clustered in the plasma membrane (Fig. 3, bottom panels). This receptor distribution was examined at higher resolution by immunogold labeling and transmission electron microscopy. In untreated cells, receptors were located prominently on microvillar extensions of the plasma membrane and were rarely observed in membrane invagina-

tions (Fig. 4*a*, arrows). In cells treated with isoproterenol at 16 °C, less receptor immunolabeling was observed on microvillar processes of the plasma membrane and more receptors were clustered within or immediately surrounding invaginations of the plasma membrane (Fig. 4*b*, arrows). Some of these invaginations appeared to be associated with an electron-dense membrane coat in osmium-stained preparations (Fig. 4, *b* and *c*), reminiscent of clathrin coated pits that mediate endocytosis of constitutively internalized receptors (19). Quantitation of this redistribution indicated that agonist treatment at 16 °C caused a 38% decrease in the number of immunogold particles associated with microvillar extensions of the plasma membrane and a 14-fold increase in the number of immunogold particles associated with membrane pits (Fig. 4, legend). Like endocytosis, receptor recycling was blocked at reduced temperature. Following agonist-induced internalization at 37 °C, recycling of receptors to the plasma membrane was not observed at either 4 or 16 °C, even after washing cells for 60 min in the presence of 100 μM alprenolol (not shown).

Agonist-independent Endocytosis of Receptors following Agonist-dependent Redistribution within the Plasma Membrane—The temperature block of receptor endocytosis could be readily reversed by warming cells to 37 °C. Following treatment of cells with isoproterenol at 4 or 16 °C, during which no receptor internalization was observed (Fig. 5, *a* and *b*), warming to 37 °C (in the continued presence of agonist) resulted in rapid receptor internalization that was indistinguishable from that observed in cells treated at 37 °C without temperature shift (Fig. 5, *c* and *d*). After agonist treatment of cells at 4 °C, subsequent receptor internalization upon warming required the continued presence of agonist. No internalization was observed in cells pretreated with agonist at 4 °C, then washed and incubated at 4 °C in the presence of excess antagonist prior to warming to 37 °C (Fig. 5*e*). In contrast, after pretreatment of cells with agonist at 16 °C, subsequent receptor internalization upon warming to 37 °C did not require the continued presence of agonist. Agonist-independent receptor internalization occurred upon warming 16 °C-pretreated cells, even after washing and incubating with excess antagonist for 45 min at 4 °C (Fig. 5*f*). We are confident that this washing interval is sufficient to remove bound agonist because it completely blocks receptor internalization in cells pretreated with agonist at 4 °C (compare Fig. 5, *e* and *f*). Maximum internalization under these conditions was observed after warming cells to 37 °C for approximately 10 min. Incubation at 37 °C with antagonist for longer than 20 min prior to fixation resulted in no detectable internalization (not shown). We believe that this is because agonist binding commits receptors to only a single round of endocytosis and recycling, which can be completed within 20 min.

Taken together, our findings suggest that the agonist-induced internalization of β₂-receptors observed by ligand binding assay, subcellular fractionation, or receptor localization in fixed cells represents a steady state distribution of receptors undergoing continuous endocytosis and recycling. Receptor endocytosis can be dissected into at least two steps. Agonist regulates an early step, which occurs at 16 °C but not 4 °C, and is associated with the redistribution of receptors into and around coated invaginations of the plasma membrane. Later step(s) of receptor endocytosis, after the agonist-induced redistribution

of receptors within the plasma membrane, do not require agonist and are ATP-dependent. Further studies will be required to determine the mechanism by which reduced temperature and ATP-depletion block the endocytosis of adrenergic receptors. It is possible that specific proteins that mediate receptor endocytosis are inhibited, or that the inhibition of adrenergic receptor internalization results from a general modification of membrane dynamics, such as a change in membrane fluidity. In either case, the present results indicate that the regulation of receptor endocytosis by agonist and the endocytosis process itself can be dissociated into distinct steps. Therefore it is possible that distinct receptor domains mediate and regulate receptor endocytosis. A candidate domain that may mediate receptor endocytosis is a conserved NPXXY motif that has been implicated in the agonist-induced sequestration of β₂-adrenergic receptors (7). This sequence is homologous to constitutive endocytosis signals (20, 21) and is present in adrenergic receptor subtypes which do not exhibit rapid agonist-induced internalization (7, 15). Consequently, additional receptor domain(s) may be required to regulate the endocytosis mechanism. These domain(s) could either enhance the association of receptors with, or retard the entry of receptors into, coated pits of the plasma membrane. This hypothesis could explain how adrenergic receptors, which exhibit agonist-regulated and subtype-selective internalization, can utilize the same endocytic machinery that mediates the constitutive endocytosis of a variety of receptors. Testing this hypothesis will require identifying these domain(s) and the cellular protein(s) that interact with them.

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