Journal of Cell Science 108, 2983-2991 (1995)
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Ligand-stimulated β_2-adrenergic receptor internalization via the constitutive endocytic pathway into rab5-containing endosomes

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SUMMARY

The small GTPase rab5 appears to be rate-limiting for the constitutive internalization of transferrin receptor and for fluid-phase endocytosis. However, it is unknown whether rab5 regulates receptors whose internalization is stimulated by the binding of ligand, and whether such receptors change the underlying rate of the endocytic pathways they utilize. As a model for ligand-stimulated endocytosis, we used transfected HEK293 cells expressing high levels of an epitope-tagged human β_2-adrenergic receptor. Nearly all receptors were on the cell surface in the absence of agonist, but within ten minutes of agonist addition >50% of receptors internalized and colocalized extensively with rab5. Hypertonic sucrose blocked β_2-adrenergic receptor internalization, as well as that of transferrin receptor, suggesting a clathrin-mediated process. In contrast, an inhibitor of potocytosis had little effect upon β_2-adrenergic receptor internalization, suggesting that this process did not require active caveolae. Consistent with this finding, caveolin was not detectable in the 12β6 line, as assessed by western blotting with a polyclonal anti-caveolin antibody. Stimulated receptor internalization did not affect the rate or capacity of the constitutive endocytic pathway since there was no detectable increase in fluid-phase endocytosis after addition of β-agonist, nor was there a significant change in the amount of surface transferrin receptor. Altogether, these data suggest that β_2-adrenergic receptors internalize by a clathrin-mediated and rab5-regulated constitutive endocytic pathway. Further, agonist-stimulated receptor internalization has no detectable effect upon the function of this pathway.

Key words: rab5, endocytosis, β_2-adrenergic receptor

INTRODUCTION

Cell-surface receptor molecules may be categorized into two types based upon their patterns of internalization. Receptors such as those for low-density lipoprotein or transferrin (TfR) internalize and recycle to the surface at essentially the same rate whether or not they are occupied by ligand (Anderson et al., 1982; Watts, 1985). In contrast, receptors that activate signal transduction pathways internalize at a much faster rate when occupied by ligand (Wiley et al., 1991), a property that serves an important regulatory function

Recent evidence implicates several ras-related GTPases in the regulation of constitutively internalizing receptors. Two of these GTPases, rab4 and rab5, localize to the endocytic compartment defined as the early (or sorting) endosome (Chavrier et al., 1990; van der Sluijs et al., 1991). Overexpression of rab5 increases the rate of TfR internalization (Bucci et al., 1992), and overexpression of rab4 accelerates the return of TfR to the cell surface (van der Sluijs et al., 1992). Conversely, suppression of rab5 function by overexpression of a GTP-binding defective, dominant negative rab5 slows the rate of TfR internalization (Bucci et al., 1992). Thus, these rab GTPases appear to be important in regulating the internalization and recycling of the TfR.

In contrast to TfR, very little is known about the relationship between rab proteins and the ligand-stimulated internalization of signal transducing receptors. One important receptor of this type is the human β_2-adrenergic receptor (β_2AR), a heptahelical G-protein coupled receptor. Upon binding to agonist, β_2AR activates adenylycyclase via G_s, then undergoes a desensitization process that includes receptor phosphorylation to uncouple it from G_s and receptor internalization into endocytic vesicles (Lohse, 1993). Upon removal of agonist, receptor rapidly returns to the surface in a fully sensitized condition (Su et al., 1980). In this study, we examine the localization of rab5 with respect to internalized β_2ARs, and present evidence that these receptors utilize the constitutive endocytic apparatus for internalization without detectably influencing global endocytic processes.

MATERIALS AND METHODS

Cells and reagents

12β6 is a G418^R line of HEK293 cells stably transfected with a cDNA encoding epitope-tagged β_2ARs (von Zastrow and Kobilka, 1992), and was a generous gift from B. Kobilka (Stanford University). The haemagglutinin (HA) epitope tag (YPYDVPDYA) is fused to the receptor amino terminus. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum

(FBS) and 250 μg/ml of G418 (Gibco/BRL). [^{3}H]CGP-12177 (Staehelin et al., 1983) (44 Ci/mmol) and [^{3}H]dihydroalprenolol (DHA) (Lefkowitz et al., 1974) (106 Ci/mmol) were obtained from DuPont-New England Nuclear. Unless otherwise specified, all other reagents were purchased from Sigma Chemical Co.

Antisera

The mouse monoclonal IgG against the HA epitope (12CA5) (Niman et al., 1983) was purchased from Berkeley Antibody Co. or Boehringer-Mannheim, and the mouse monoclonal IgG against human TfR, from Amersham. Rabbit polyclonal antibodies against human caveolin were purchased from Transduction Laboratories. A rabbit antiserum against a peptide representing the fifteen C-terminal amino acids of the β_2AR was a gift from Dr B. Kobilka (von Zastrow and Kobilka, 1992). To raise antibody against rab5, a *rab5* cDNA (Zahraoui et al., 1989) was cloned into the T7-expression vector pT7.7, expressed in *Escherichia coli* BL21(DE3) (Studier et al., 1990) and purified to 95% homogeneity by sequential column chromatography (Hoffenberg et al., 1995). The rab5 protein was injected into rabbits to obtain a polyclonal antiserum, and total IgG was purified from crude serum by passage through DEAE Affi-Gel Blue (Bio-Rad). Specific antibody was obtained by the binding of IgG to rab5 protein immobilized on Affi-Gel 10 (Bio-Rad), followed by elution with 3 M $MgCl_2$ and desalting by gel filtration. The secondary antibodies were FITC-conjugated sheep anti-mouse IgG and Texas Red-conjugated donkey anti-rabbit IgG, both purchased from Amersham. The anti-rab5 antiserum recognizes one major band in a western blot of 12β6 whole cell extracts, and does not cross-react with rab4, another ras-related small GTPase that localizes to the early endosome (van der Sluijs et al., 1991) (data not shown).

β_2AR radioligand binding assays

12β6 cells growing in 100 mm dishes were incubated with 0.1 mM ascorbic acid with or without 5 μM isoproterenol (ISO) for the indicated times, then harvested by trituration with ice-cold Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (PBS) containing 5 mM EDTA. The cells were washed three times by suspending in ice-cold DMEM with 20 mM HEPES, pH 7.4 (DMEM-HEPES) and centrifuging at 1,000 ***g*** for 5 minutes. Binding of the tritiated ligands was performed in DMEM-HEPES with whole cells and various concentrations of radioligand (0.05-2 nM) in a total volume of 0.5 ml. Incubation with [^{3}H]CGP12177 was with 50 μg of whole cells at 4°C for 16 hours, and incubation with [^{3}H]DHA was with 25 μg of whole cells at 37°C for 2 hours. Bound ligand was measured by filtration through glass fiber filters (GF/C, Whatman) and scintillation counting of bound ligand. Nonspecific binding of both radioligands was determined by additional incubations in the presence of 3 μM alprenolol. Mean counts from triplicate measurements were analyzed by least squares regression fitting of Scatchard transformations using the program LIGAND (Munson and Rodbard, 1980). In some experiments, an estimate of B_{max} was obtained by incubation as above in triplicates with 3 nM [^{3}H]CGP12177. Protein determinations were performed using the Coomassie Plus reagent (Pierce Chemical Co.). In some experiments, cells were exposed to 0.45 M sucrose in DMEM-HEPES for 30 minutes, or to medium with 10^{-7} M 4β-phorbol 12-myristate 13-acetate (PMA) for 15 minutes prior to the addition of ISO to 5 μM for 15 minutes.

Measurement of fluid-phase endocytosis

12β6 cells were cultured in DMEM + 10% FBS on poly-L-lysine coated 6-well plates until 60-80% confluent. The cells were washed

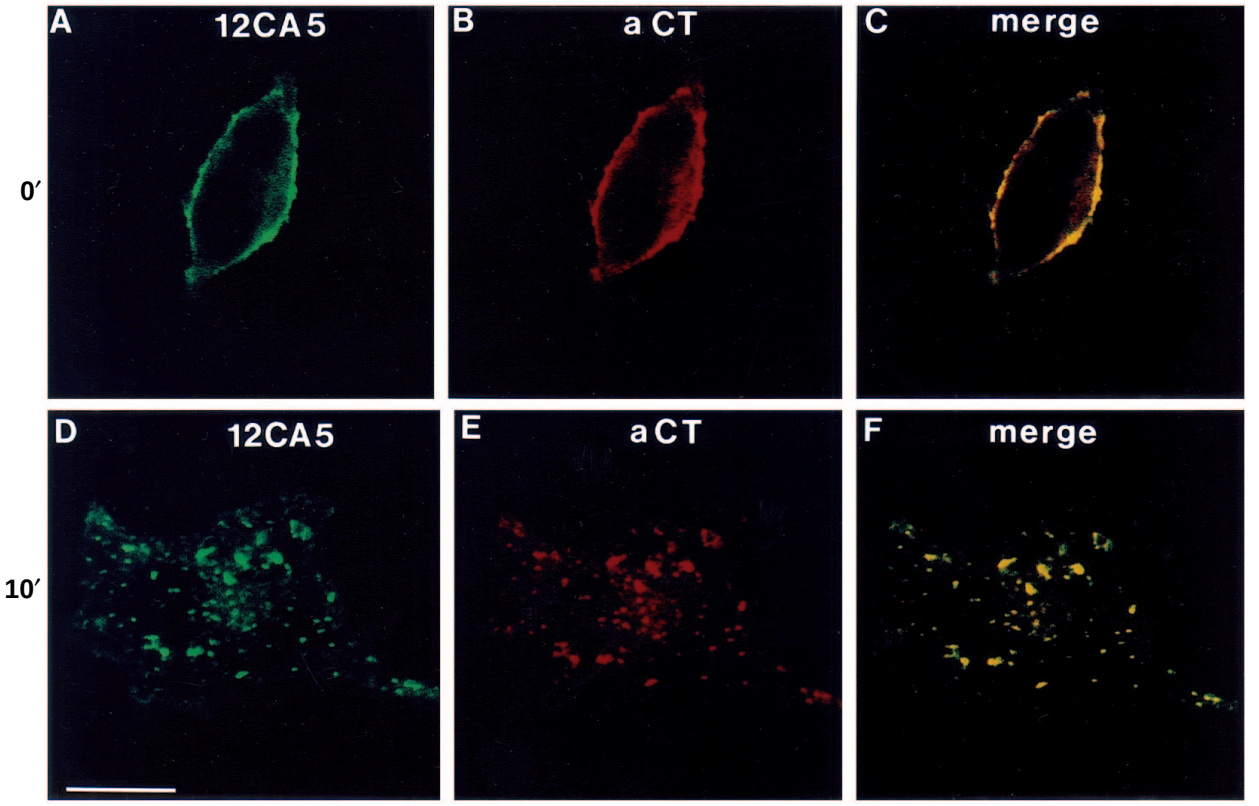

Fig. 1. Colocalization of β_2AR epitopes. 12β6 cells were exposed to ISO for 10 minutes (10′), or not treated (0′), then prepared for confocal microscopy as described in Materials and Methods. (A-C) Untreated cells; (D-F) ISO-treated cells. (A and D) 12CA5 antibody; (B and E) anti-C-terminal peptide antibody; (C and F) mergers of A and B and D and E, respectively. The yellow color indicates coincident green and red within a pixel. Bar, 10 μm.

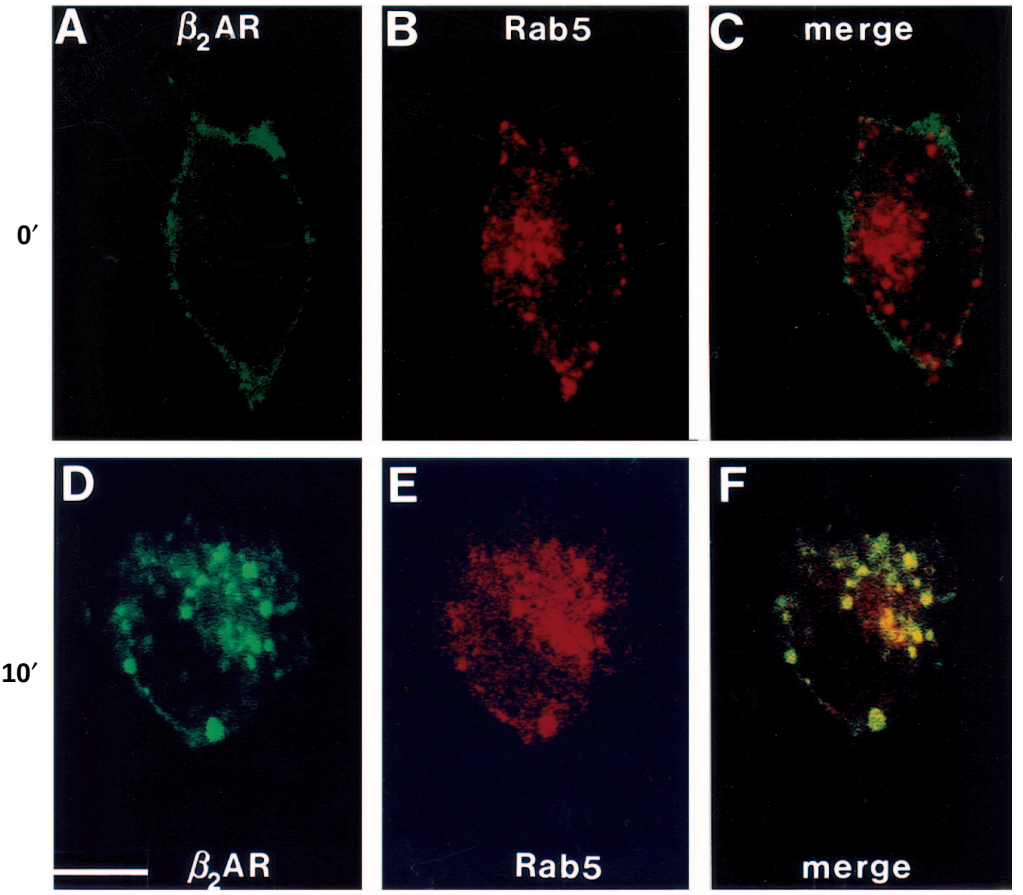

Fig. 2. Colocalization of rab5 and β_2ARs. 12β6 cells were exposed to 5 μM ISO for 10 minutes (10′), or not treated (0′), then prepared for confocal microscopy as in Fig. 1. (A-C) Untreated cells; (D-F) ISO-treated cells. (A and D) β_2ARs; (B and E) rab5; (C) computer-aided merger of A and B; (F) computer-aided merger of D and E. Bar, 10 μm.

once with DMEM-HEPES, and once with DMEM-HEPES with 1% BSA, then exposed to DMEM-HEPES with 1% BSA and 1 mg/ml horseradish peroxidase at 37°C, with or without 5 μM ISO. At various times, the plates were quickly immersed in PBS with 1% BSA: once at 37°C, and twice at 4°C. The monolayers were then washed five times with PBS + 1% BSA (five minutes per wash). The cells were solubilized in 0.1% Triton X-100, and the lysates assayed for HRP activity exactly as described (Nolan, 1992). The activity is expressed as ng HRP/mg cell protein accumulated at each time point.

Immunocytochemistry

Cells growing on poly-L-lysine coated #1 glass coverslips were exposed to 5 μM isoproterenol with 0.1 mM ascorbic acid for various times, then washed three times with PBS and fixed with freshly prepared 4% paraformaldehyde. Specimens were then washed and incubated in a 0.34% L-lysine, 0.05% Na-m-periodate solution to block the remaining reactive aldehyde groups. Cells were permeabilized with 0.2% Triton X-100 in PBS, followed by blocking with 10% heat-inactivated goat serum (HIGS) in PBS. Primary antibodies, diluted in 0.2% HIGS, 0.05% Triton X-100 in PBS, were added and

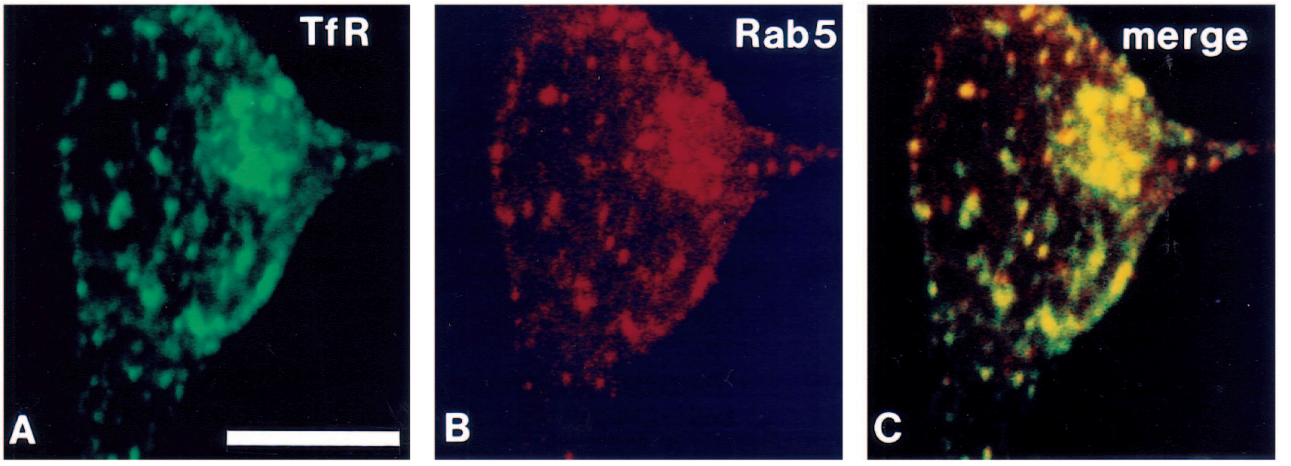

Fig. 3. Colocalization of rab5 and TfR. 12β6 cells were exposed to 5 μM ISO for 10 minutes, then prepared for confocal microcopy as in Fig. 1. (A) TfR; (B) rab5; (C) computer-aided merger of A and B. Bar, 10 μm.

left on the cells for 16 hours. The monoclonal anti-HA (12CA5) and anti-TfR antibodies were diluted 1:100. The polyclonal anti-rab5 antibody was diluted 1:100, and the polyclonal anti-β_2AR-C-terminal antiserum was diluted 1:200. After three washes in PBS, the secondary antibodies (diluted at 1:100 in the same buffer as the primary antibodies) were applied and incubated in darkness for 1 hour. The specimens were again washed three times in PBS, then mounted with Mowiol and allowed to dry overnight in darkness. Images were acquired using a Bio-Rad MRC500 confocal imaging system attached to a Zeiss Axiovert 100 microscope, and then printed on a Sony color video printer.

Flow cytometry

12β6 cells were harvested from monolayers as described above, then suspended in DMEM-HEPES at 37°C. One-half of the cells were exposed to 5 μM ISO for 15 minutes, and the other half were left untreated. The cells were quickly chilled, then washed by centrifugation and resuspension in PBS. The cells were incubated for 60 minutes on ice with 1 μg/ml of either 12CA5, monoclonal anti-TfR, or with nonspecific mouse IgG. After washing, the cells were incubated with 1 μg/ml of FITC-anti-mouse IgG for 60 minutes on ice, then washed again and analyzed by flow cytometry. This was performed on an Epics Profile I (Coulter Cytometry, Hialeah, FL) with an argon laser tuned to 488 nm and with three fluorescence channels. Green (FL1) fluorescent events were accumulated on a 4 decade logarithmic scale.

Western blotting

12β6 cells and human endothelial cells were washed three times with PBS, then lysed by boiling in 50 mM Tris-HCl (pH 6.8), 100 mM β-mercaptoethanol, 2% SDS, 10% glycerol. The whole cell extracts were electrophoresed through a 12% polyacrylamide-SDS gel in a Tris-tricine buffer (Schagger and von Jagow, 1987), then electroblotted onto a PVDF filter (Immobilon, Millipore). The filter was blocked by incubation in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Tween-20 (TBST) with 5% BSA, incubated with primary anti-caveolin antibody (1:100 dilution) in TBST, washed and then incubated with a 1:2,000 dilution in TBST of goat anti-rabbit IgG conjugated to HRP. After washing, the blot was processed for chemiluminescent detection using the Amersham ECL kit.

RESULTS

Colocalization of β_2ARs with rab5

Previous studies have colocalized rab5 in the endocytic compartment with the constitutively internalizing TfR (Bucci et al., 1992). To determine whether rab5 also would colocalize with a receptor whose internalization is ligand-stimulated, a two-color imaging technique was used. The utility of this colocalization technique is illustrated in Fig. 1. 12β6 cells were stained with the monoclonal antibody (12CA5) against the extracellular HA epitope tag on the β_2AR and with an anti-β_2AR C-terminal peptide antiserum. Colocalized polyclonal and monoclonal antibodies should produce a yellow staining pattern in the merged image, which is the result expected here since both antibodies stain the same receptor molecule. In untreated cells, surface staining with both antibodies was observed (Fig. 1A,B), and a merger of the images produced the expected yellow pattern (Fig. 1C). Coincidence of staining also resulted when cells treated with ISO for 10 minutes were examined: identical patterns of vesicular staining were observed (Fig. 1D,E), which produced a yellow pattern when the images were merged (Fig. 1F).

This two-color imaging procedure then was used to colocalize β_2ARs with rab5. In untreated cells the staining with 12CA5 and the anti-rab5 antibody showed very little overlap (Fig. 2A,B,C). The anti-rab5 antibody gave a punctate pattern of intracellular staining (Fig. 2B), similar to that previously observed in BHK cells using a rab5 antiserum raised against a peptide derived from the rab-isotype-specific carboxyl-terminal region of rab5 (Bucci et al., 1992). A merger of these images (Fig. 2C) indicated little or no overlap of staining. Treatment with ISO for 5 minutes appeared to result in a decrease of surface β_2ARs (data not shown), then after 10 minutes β_2ARs appeared concentrated within distinct intracellular vesicles (Fig. 2D). A merger of Fig. 2D and E (Fig. 2F) indicated that internalized β_2ARs were almost completely colocalized with rab5, as shown by the extensive yellow color of the punctate vesicles. Similar results were obtained with 30 minutes of ISO treatment (data not shown). Thus, β_2ARs moved into early endosomes (as defined by rab5 colocalization) shortly after binding to agonist, and remained there for at least 30 minutes of agonist exposure. Internalized β_2ARs also colocalized extensively with transferrin receptor (data not shown), as reported earlier (von Zastrow and Kobilka, 1992).

TfR and rab5 have been colocalized in other cell types, but not previously in cells derived from the HEK293 line. More significantly, it was important to determine whether treatment of the 12β6 line with agonist caused a differential redistribution of TfR and rab5. Thus, dual immunolocalization of these proteins was performed on 12β6 cells that had been treated with ISO for 10 minutes. The images in Fig. 3 show extensive colocalization of TfR and rab5, with only a few vesicles that appear to be positive only for one or the other. There was no apparent redistribution of rab5 or transferrin receptor in response to ISO treatment (data not shown). In addition, no redistribution of rab5 between particulate and cytosolic fractions was detectable by immunoblotting (data not shown),

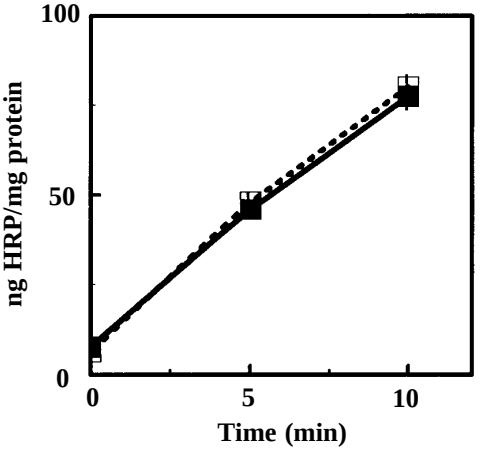

Fig. 4. The effect of agonist upon fluid-phase endocytosis. 12β6 cells were incubated at 37°C in DMEM-HEPES containing 1 mg/ml BSA and 1 mg/ml HRP for the indicated times in the presence (open squares) or the absence (closed squares) of 5 μM ISO. The cells were then washed, harvested and assayed for cell-associated HRP as described in Materials and Methods. The points are the averages of three HRP assays, ± s.d.

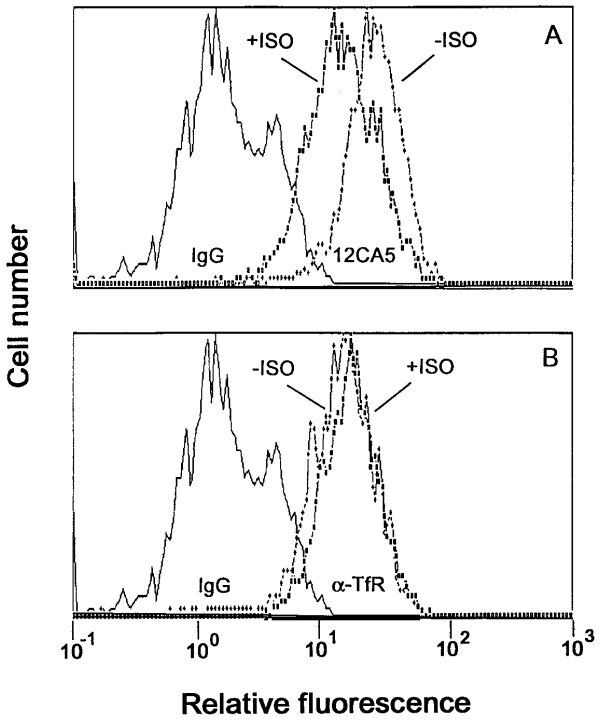

Fig. 5. Flow cytometry of β_2AR and TfR internalization. 12β6 cells were treated with ISO for 15 minutes, or left untreated, then harvested and processed for flow cytometry as described in Materials and Methods. 3-4×10^3 cells were analyzed, and the distribution of fluorescence intensities recorded over 256 channels. (A) β_2AR; (B) TfR.

in contrast to the induced redistribution of rab4 (Cormont et al., 1993) and rab3A (Fischer von Mollard et al., 1991) observed in other systems.

The effect of agonist on fluid-phase endocytosis and transferrin receptor distribution

The colocalization of β_2ARs with TfR and rab5 suggests that β_2ARs enter the constitutive, clathrin-mediated endocytic pathway after binding agonist. To determine whether the rate or capacity of this pathway was changed by the agonist-stimulated internalization of β_2ARs, the initial rate of fluid-phase endocytosis was measured immediately after the addition of agonist. By restricting the analysis to early time points (0-10 minutes), the effects of receptor activation of signal transduction pathways on vesicular transport (Eker et al., 1994) should be minimized. These measurements indicated that fluid-phase endocytosis (as measured by the internalization of horseradish peroxidase) was similar in both control and ISO-treated cells (Fig. 4).

The above result argues against an immediate effect of β_2AR internalization upon fluid-phase endocytosis. To determine whether the synchronous internalization of large numbers of β_2ARs would affect the distribution of a constitutively cycling receptor, flow cytometry was used to measure the amount of TfR on the surface of 12β6 cells in the presence and absence of β-agonist. Control 12β6 cells, or cells treated with ISO for 15 minutes, were washed and incubated first with a monoclonal antibody against TfR, HA (12CA5) or a nonspecific IgG. After a second incubation with FITC-anti IgG, flow cytometry was

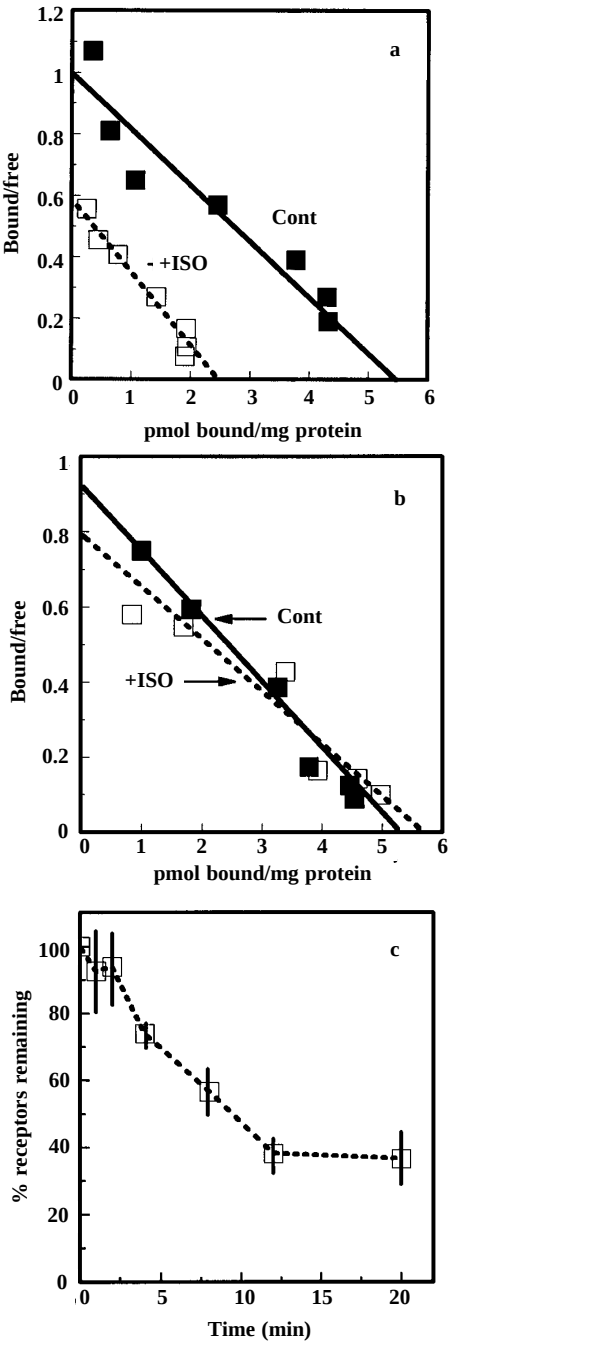

Fig. 6. Internalization of β_2AR in 12β6 cells. (a) Surface receptors. Cells were treated with 5 μM ISO for 15 minutes at 37°C, then harvested, washed and assayed for surface receptors using the hydrophilic radioligand [^{3}H]CGP12177 as described in Materials and Methods. The binding data are analyzed by a Scatchard plot. Open squares, ISO-treated cells; closed squares, untreated cells. (b) Total receptors. Cells were exposed to ISO as for a, then harvested and assayed for total receptors using the hydrophobic radioligand [^{3}H]DHA as described in Materials and Methods. Open squares, ISO-treated cells; closed squares, untreated cells. (c) Kinetics of β_2AR internalization. 12β6 cells were incubated with 5 μM ISO for various times intervals, then chilled to 0°C and harvested as described for a. Surface receptor was assayed by incubating the washed cells in triplicate with 3 nM (10× K_d) [^{3}H]CGP12177 at 0°C overnight, followed by filtration to determine bound ligand. The points are the average of three assays ± s.d.

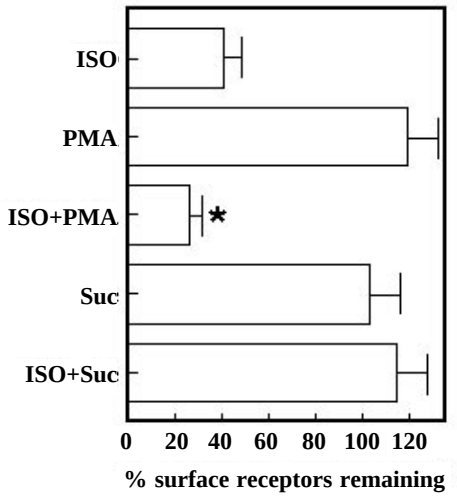

Fig. 7. The effects of inhibitory treatments on the internalization of β_2ARs. 12β6 cells were incubated with PMA (10^{-7} M) for 15 minutes at 37°C prior to the addition of ISO to 5 μM for 15 minutes. For sucrose inhibition, cells were incubated in the presence of 0.45 M sucrose for 30 minutes prior to the addition of 5 μM ISO for 15 minutes. Cells were harvested and assayed for surface receptor as described in Fig. 6C. The percentages shown are relative to cells that had received no treatment (or vehicle) relative to treated cells. Asterisk (*) denotes significant difference (P<0.05) from cells treated with ISO alone.

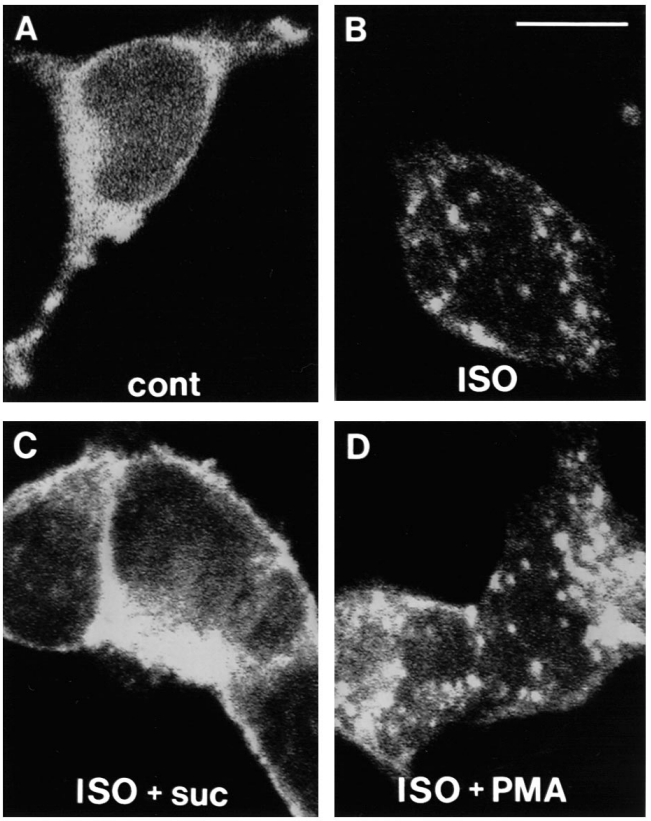

Fig. 8. Confocal microscopy of β_2ARs in cells treated with inhibitory agents. 12β6 cells were treated with 5 μM ISO for 15 minutes, in the presence or absence of inhibitory agents as described in Fig. 7, then stained for β_2AR and prepared for confocal microscopy as in Fig. 1. (A) Untreated; (B) ISO treated; (C) ISO treated in hypertonic sucrose; (D) ISO treated, with PMA. Bar, 10 μm.

performed (Fig. 5). Under conditions where there was substantial internalization of β_2ARs, there was little detectable change in the number of cell-surface TfRs. The fluoresence intensity mean values for 12CA5 binding (after subtraction of background fluorescence resulting from nonspecific IgG) were 19.42 for control cells, and 7.71 for ISO-treated cells (39.7% of control), numbers that agree well with those obtained from radioligand binding assays (see below).

Pathway of β_2AR internalization

To distinguish among possible pathways of β_2AR internalization, 12β6 cells were treated with various inhibitors of endocytosis and then with ISO prior to assay for surface receptors. First, we examined specific parameters of the ligand-binding assay used for this cell line. Receptors were assayed by incubation with the hydrophilic radioligand [^{3}H]CGP12177, which detects only surface receptors (Staehelin et al., 1983), and with the hydrophobic ligand [^{3}H]DHA, which detects both surface and internal receptors (Mahan et al., 1985b). In untreated cells, both radioligands detected similar numbers of receptors (≈337,000/cell), indicating that the great majority of receptors were localized to the cell surface. Thus, despite the large numbers of receptors compared with a more physiological level of expression (1,000-1,500 sites/cell) (Mahan et al., 1985a), there appeared to be no large internal pool of receptors in untreated cells. When cells were exposed to 5 μM ISO for 15 minutes, there was a loss of nearly 60% of receptors from the cell surface (Fig. 6a). Agonist treatment for this brief time did not cause a loss of total cellular receptors, since similar receptor numbers were found in control and in ISO-treated cells when assayed with the hydrophobic radioligand [^{3}H]DHA (Fig. 6b). Internalization was essentially complete by 10 minutes, and exhibited a $t_{\frac{1}{2}}$ of ≈5 minutes (Fig. 6c). A similar degree of internalization, with similar kinetics, was observed using mouse L-cells stably expressing non-epitope-tagged human β_2ARs at a level of 300,000 receptors/cell (data not shown). In 12β6 cells, the K_d of [^{3}H]CGP12177 at 0°C in both untreated and agonist-treated cells was approximately 0.3 nM. Thus, in subsequent assays we estimated B_{max} using a single concentration of 3 nM to ensure saturation of receptors.

Earlier reports suggested that a proportion of the β_2ARs in A431 cells is found localized to plasma-membrane associated smooth vesicles, and that internalization induced by the binding of an anti-β_2AR antibody did not involve coated pits or vesicles (Raposo et al., 1992). To further explore this question, we asked whether the internalization of β_2ARs would be inhibited by a prior incubation of cells with hypertonic sucrose (Daukas and Zigmond, 1985), a treatment that inhibits the formation of functional clathrin-coated pits (Heuser and Anderson, 1989). When 12β6 cells were exposed to ISO in the presence of hypertonic sucrose, internalization of β_2AR, as assayed by radioligand binding, was blocked completely (Fig. 7). Confocal microscopy showed that in the presence of hypertonic sucrose, β_2ARs in ISO-treated cells remained localized to the cell surface (Fig. 8). The effectiveness of the sucrose treatment in disrupting clathrin-mediated endocytosis was revealed by confocal microscopy to determine TfR cytolocal-

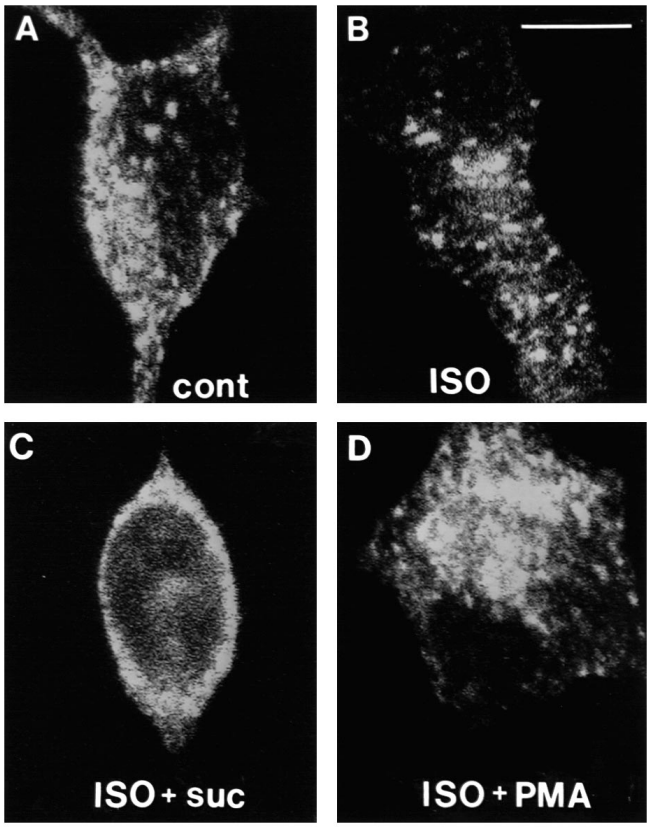

Fig. 9. Confocal microscopy of TfR in cells treated with inhibitory agents. 12β6 cells were treated with 5 μM ISO for 15 minutes, in the presence or absence of inhibitory agents as described in Fig. 7, then stained for TfR and prepared for confocal microscopy as in Fig. 1. (A) Untreated; (B) ISO treated; (C) ISO treated in hypertonic sucrose; (D) ISO treated, with PMA. Bar, 10 μm.

ization. In control cells, TfR was localized mainly within intracellular vesicles, as has been observed previously (Bucci et al., 1992). After treatment with hypertonic sucrose for a period of time long enough to allow most TfR to return to the surface, the failure of subsequent TfR internalization was indicated by a predominant cell surface staining (Fig. 9).

Because a colocalization of internalized β_2AR with VIP21/caveolin has been reported in A431 cells (Dupree et al., 1993), and others have shown an association between caveolae and some signalling receptors (Sargiacomo et al., 1993), the possibility that β_2AR might internalize by this pathway in 12β6 cells was investigated. Disruption of caveolae can be achieved by treatment of cells with phorbol esters (Smart et al., 1994). In the 12β6 line, treatment with 10^{-7} M PMA did not block the internalization of β_2ARs, but instead there was a small stimulatory effect (Fig. 7). This result is consistent with previous studies of other cell lines, where phorbol ester treatment was found to increase the rate of TfR internalization (Schonhorn et al., 1995). The level of PMA used in our experiments was sufficient to activate protein kinase-C at least two-fold, as assayed by the phosphorylation of an oligopeptide substrate (data not shown). The apparent lack of inhibition of internalization of either β_2ARs or TfRs by PMA was verified by confocal microscopy (Fig. 9).

As a further control on the effect of PMA on caveolae, an attempt was made to assay a specific caveolar function. In at least some cell types, receptors for folate are concentrated within caveolae (Anderson, 1993a,b), and the binding of [^{3}H] folic acid is inhibited by PMA treatment (Smart et al., 1994). However, 12β6 cells did not detectably accumulate [^{3}H] folic acid (data not shown), thus we were unable to demonstrate a specific effect of PMA on caveolar function. To investigate the possibility that 12β6 cells lack caveolae, we performed a western blot on whole cell extracts using a rabbit polyclonal antibody against human caveolin. As a positive control, an extract of human endothelial cells was used. As shown in Fig. 10, 12β6 cells appear to have little or no caveolin. The antibody detects a protein of a similar molecular mass, however, this signal is not significantly higher than background.

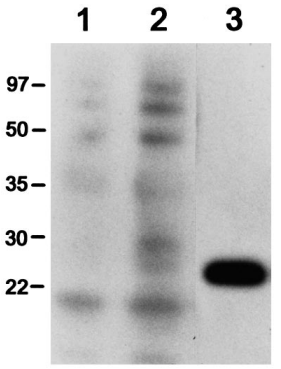

Fig. 10. Western blotting of 12β6 cell extracts with anti-caveolin antibodies. Whole-cell extracts of 12β6 and human endothelial cells were electrophoresed on a 12% SDS-polyacrylamide gel, then electroblotted to an Immobilon filter and probed with a rabbit polyclonal antiserum to human caveolin as described in Materials and Methods. Lane 1, 2 μg of 12β6 extract; lane 2, 5 μg of 12β6 extract; lane 3, 2 μg of human endothelial cell extract. Size standards (kDa) are shown on the left.

DISCUSSION

There are several major conclusions to be drawn from the data presented here. Agonist-bound β_2ARs appeared to internalize by a clathrin-mediated pathway, after which receptor rapidly colocalized with the regulatory GTPase rab5 in the early endosomal compartment. The agonist-stimulated endocytosis of overexpressed β_2AR appeared to have no effect upon other endocytic processes, either directly or indirectly. Of general significance, the behavior of the 12β6 line resembles that of cells expressing low numbers of native β_2AR derived directly from airway smooth muscle or epithelium (Hall et al., 1993; Nogami et al., 1993) or by transfection of immortalized fibroblasts (Barak et al., 1994). Similar to these other models, β_2ARs in the 12β6 line are uncoupled from adenylycyclase following a short agonist exposure, and after removal of agonist, receptor is resensitized and recycled to the surface (K. J. Morrison et al., unpublished).

The ras-related GTPase rab5 previously has been shown to be located specifically in early endosomes (Chavrier et al., 1990), and thus our data suggests that the great majority of internalized β_2ARs were limited to this compartment and not to the late endosome or *trans*-Golgi network. Because rab5 is

known to be rate-limiting for the internalization of TfR and for fluid-phase endocytosis (Bucci et al., 1992), an important implication of our finding is that rab5 also may be rate-limiting in the ligand-induced internalization of a signalling receptor, thereby extending the range of receptor-mediated endocytic events regulated by rab5.

The ligand-induced internalization of β_2ARs exhibited a $t_{\frac{1}{2}}$ of about 5 minutes, and was almost complete by 10 minutes after the addition of agonist (Fig. 6c). These kinetics are quite similar to the rate at which TfR is internalized (Watts, 1985), and is consistent with our other data suggesting that β_2ARs simply enter the constitutive endocytic pathway after ligand binding, with no induction of a distinct endocytic mechanism or acceleration of the constitutive pathway. That β_2AR internalization occurs by clathrin-coated vesicles is supported by the finding that internalization was completely blocked by hypertonic sucrose, a treatment that disrupts the formation of functional clathrin lattices (Heuser and Anderson, 1989). We cannot completely exclude the possibility that β_2ARs internalize by a non-clathrin pathway that merges with early endosomes and that does not involve significant fluid phase uptake. Such a pathway would have to be inhibitable by hypertonic sucrose, and would have to occur with kinetics very similar to clathrin-mediated endocytosis. During the preparation of this manuscript, an electron microscopic study of 12β6 cells showed that about 18% of β_2ARs were located within coated pits following agonist treatment (von Zastrow and Kobilka, 1994). This lower number of β_2ARs within coated vesicles compared with the >60% internalization that we observe could be due to difficulties in identifying coated structures in micrographs, the rapid transit of β_2ARs through coated structures, or to the occurrence of fixation or sectioning artifacts. The complete inhibition of β_2AR internalization by hypertonic sucrose that we observe strongly suggests that those β_2ARs observed within coated pits were indeed intermediate structures representative of the whole receptor population.

Consistent with the idea that β_2ARs passively entered the constitutive endocytic pathway in agonist-treated cells is the finding that the simultaneous endocytosis of large numbers of β_2ARs had no detectable effect on fluid-phase endocytosis (Fig. 4), on the subcellular distribution of rab5 (Figs 2 and 3), or on the amount of surface TfR (Fig. 5). The lack of change in rab5 distribution stands in apparent contrast to several other rab proteins, which cycle on and off membranes as a function of induced vesicle fusion. For example, rab3A dissociates from synaptic vesicles during exocytosis (Fischer von Mollard et al., 1991), and rab4 dissociates from vesicles carrying the glucose transporter (GLUT4) when induced to fuse with the plasma membrane (Cormont et al., 1993). The failure to detect an agonist-induced redistribution of rab5 is consistent with the notion that ligand-binding by β_2ARs does not result in an alteration of constitutive endocytosis, but rather makes the β_2ARs available to the endocytic apparatus.

In contrast to these findings, a previous study of A431 cells suggested that β_2ARs internalize through noncoated vesicles. When receptor internalization is induced by the binding of an anti-β_2AR monoclonal antibody, receptor initially associates with noncoated membrane invaginations, which after 30 minutes form 'fused vesicles'. By 60-90 minutes, β_2ARs eventually move into lysosomes and multivesicular bodies (Raposo et al., 1992). In our studies, the kinetics of internalization were much more rapid, being essentially complete by 10 minutes (Fig. 6c), whereas in A431 cells, the same degree of internalization requires 30 minutes (Raposo et al., 1992). In the same cell line, under these conditions, β_2ARs also appear to colocalize with caveolin (Dupree et al., 1993), a major component of caveolae (Anderson, 1993a,b). These are small, flask-shaped plasmalemmal structures wherein GPI-anchored membrane proteins and possibly cell surface signalling receptors are believed to be concentrated (Sargiacomo et al., 1993). In the 12β6 line, the internalization of β_2ARs still occurred under conditions of phorbol ester treatment, which should disrupt caveolae (Fig. 7), and no association of receptors with caveolae was reported in a recent electron microscopic study of 12β6 cells (von Zastrow and Kobilka, 1994). Our data also show that 12β6 cells contain very little if any caveolin, as measured by western blotting with a polyclonal antiserum (Fig. 10). That caveolae are not necessarily ubiquitous was shown recently by Scherer et al. (1994), who examined diverse mouse tissues by northern and western blotting. Interestingly, mouse kidney contained no detectable caveolin, consistent with the origin of the parent cell line of the 12β6 line, human embryonic kidney. The apparent discrepancy of our results with those previously obtained with A431 cells could be due to differences in the cell lines employed, or it is possible that antibody-bound β_2ARs follow a different intracellular itinerary than receptors which have bound agonist. Receptors cross-linked with antibody may also form an artefactual association with caveolae, as has been reported for other cell-surface proteins (Mayor et al., 1994).

The β_2ARs in 12β6 cells are unlikely to be endocytosed by macropinocytosis. This process has been studied in A431 cells, where fluid-phase endocytosis is induced 6-10-fold by treatment with epidermal growth factor (West et al., 1993), however there are no reports of receptors specifically internalizing through macropinosomes. Treatment of 12β6 cells with agonist did not detectably stimulate fluid-phase endocytosis (Fig. 4), indicating that macropinocytosis was not occurring under our experimental conditions. Also, β_2ARs colocalized with rab5 (Fig. 2), whereas fluid-phase markers taken up by stimulated macropinocytosis do not pass through endosomes (Hewlett et al., 1994).

If β_2ARs utilize the constitutive endocytic pathway, then this receptor should contain amino acid motifs that are known from studies of other receptors to be important for internalization and targeting to the endosome. The β_2AR contains such an amino acid motif, located within the C-terminal cytoplasmic domain and presumably juxtaposed to the plasma membrane (Barak et al., 1994). Within this sequence is a critical tyrosine residue embedded within an NPXXY motif which is related to one type of signal required for the internalization of several constitutively internalizing receptors (Sandoval and Bakke, 1994). In addition to this signal, extensive mutational studies have implicated several other regions of the β_2AR cytoplasmic C terminus in the regulation of its endocytosis, including serine residues that may be phosphorylated by the βAR kinase in response to agonist (Yu et al., 1993). Thus, while β_2ARs appear to utilize the same endocytic pathway as constitutively internalizing receptors, the interaction of β_2ARs with the endocytic apparatus may be regulated by distinct structural elements of the receptor, which render it accessible to the endocytic apparatus only upon binding with agonist.

We thank Becky Scott for help with the confocal microcopy, and Drs J. Mathew and J. Stasek for the protein kinase-C assay. This work was supported by NIH grants HL43161, HL50047, NS 32583 and AI 28071, a VA Merit Review grant, and by a grant from the American Heart Association, Texas Affiliate.

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(Received 15 February 1995 - Accepted 1 June 1995)