Trafficking of β2-adrenergic receptors: insulin and β-agonists regulate internalization by distinct cytoskeletal pathways

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Summary

Insulin and β-adrenergic agonists stimulate a rapid phosphorylation and sequestration of the β2-adrenergic receptors (β2ARs). Although the expectation was that a common pathway would be involved in the trafficking of the β2AR in response to either hormone, studies reported herein show the existence of unique cytoskeletal requirements for internalization/recycling of G-protein-coupled receptors, such as the β2AR. Treatment of human epidermoid carcinoma A431 cells with nocodazole, which binds tubulin monomer in vivo and catalyzes the depolymerization of microtubules, effectively blocks β-adrenergic agonist-induced, but not insulin-induced, sequestration of β2ARs. Treatment with latrunculin-A, an agent that sequesters actin monomer and leads to loss of actin filaments, had no effect on the ability of β-adrenergic agonists to stimulate internalization of β2ARs, but blocked the ability of insulin to stimulate counterregulation of β2ARs via internalization. Although nocodazole had no effect on insulin-stimulated sequestration of β2ARs, the recycling of the internalized receptors to the cell membrane was sensitive to depolymerization of microtubules by this agent. Latrunculin-A, by contrast, blocks the recycling of β2ARs internalized in response to β-agonist, while attenuating recycling of receptors internalized in response to insulin stimulation. These data show the existence of unique cytoskeletal requirements for G-protein-coupled-receptor trafficking in response to agonist compared with a counterregulatory hormone, and for sequestration versus recycling of the receptors to the cell membrane.

Key words: G-protein-coupled receptors, Internalization, Counterregulation, Insulin, Agonist-induced, Trafficking.

Introduction

β2-Adrenergic receptors (β2ARs) are members of the superfamily of G-protein-coupled receptors (GPCRs). These prototypic GPCRs display acute homologous desensitization in response to β2-adrenergic agonists as well as counterregulation by several growth factor receptors with intrinsic tyrosine kinase activity, including insulin (Morris and Malbon, 1999). The trafficking of the β2AR is central to both agonist-induced and insulin-induced regulation of β-adrenergic signaling (Lefkowitz, 1998; Morris and Malbon, 1999). Sequestration of β2AR in response to insulin represents a loss of the surface complement of receptors and is one of the mechanisms of the counterregulatory effects of insulin on catecholamine action (Karoor et al., 1998). Agonist-induced sequestration of β2ARs has been reported using a variety of techniques (Carman and Benovic, 1998; Gagnon et al., 1998). By comparison, little is known about the counterregulation by tyrosine kinases and how insulin influences GPCR trafficking.

β2ARs are phosphorylated by insulin treatment of cells (Karoor and Malbon, 1998). Studies in vivo have shown that insulin stimulates the phosphorylation of two major tyrosine residues, Y350 and Y364; both residues are located in the C-terminal cytoplasmic domain of the β2AR (Karoor et al., 1995). The phosphorylation of the Y350 residue in response to insulin creates an SH2-binding site to which Grb2, the p85 catalytic domain of phosphatidylinositol 3-kinase, and the GTPase dynamin can dock (Shih and Malbon, 1998). Purified insulin receptor and recombinant β2ARs have been used to show that insulin stimulates the insulin receptor-catalyzed phosphorylation of these same residues (Baltensperger et al., 1996; Doronin et al., 2000). Phosphorylation of the β2AR impairs its ability to signal to its cognate G-protein Gs, a blockade that requires Grb2 with an intact SH2 domain (Shih and Malbon, 1998).

Insulin catalyzes a robust internalization of β2AR. Insulin thereby suppresses β-adrenergic signaling, precluding access of β-agonist to the β2AR. In spite of similarities in the ability of β-agonists and insulin to stimulate sequestration of the β2AR, important differences may exist in the character of the pathways by which these sequestrations occur (Karoor et al., 1998). For example, β2AR internalization in response to insulin, but not β-adrenergic agonist, can be blocked with inhibitors of phosphatidylinositol 3-kinase (PI3-kinase), such as wortmannin or LY294002 (Wang et al., 2000), as well as by inhibitors of Src activity (Shumay et al., 2002). In the current work, we extend the studies on GPCR trafficking and probe the role of cytoskeletal elements in the trafficking of β2AR by insulin and by β-agonists. The results provide compelling

evidence that insulin and β-adrenergic agonists employ unique cytoskeletal elements in trafficking receptor from the cell surface. Furthermore, the results reveal that the internalization and recycling aspects of receptor trafficking in response to insulin have unique cytoskeletal requirements: sequestration requires an intact actin cytoskeleton, whereas recycling to the cell surface requires intact microtubules.

Materials and Methods

Materials

The plasmid encoding the enhanced-green fluorescent protein (GFP)-tagged human β_2AR (in pCDNA3) was obtained from Jeffrey Benovic (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA). To analyze and characterize an expression of GFP-tagged β_2AR, the following antibodies were used: anti-β2AR [CM 02], anti-peptide antibody to an exofacial domain of the β_2AR (Wang et al., 1989) and anti-GFP rabbit polyclonal antibodies (Quantum Biotechnologies, Montreal, Quebec, CN); goat, anti-rabbit antibody conjugated with horseradish peroxidase and goat anti-mouse antibody conjugated with horseradish peroxidase (both from Kirkegard & Perry Laboratories, Gathesburg, MD); mouse anti-β-tubulin, anti-β-tubulin-Cy3 and anti-actin antibody (Sigma, St Louis, MO).

Cell culture

Human epidermoid carcinoma cells (A431) were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin (60 μg/ml) and streptomycin (100 μg/ml), and grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. A431 clones stably expressing the GFP-tagged human β_2AR were cultivated with the addition of G418. Chinese hamster ovary (CHO) cells were obtained from the ATCC collection, propagated and stably transfected with pCDNA3 harboring the GFP-tagged human β_2AR (Liu et al., 1999a; Liu et al., 1999b).

Immunoprecipitation and immunoblotting

For most studies, A431 cells were serum starved overnight with the following treatment as indicated. Cells were harvested and lysed in a lysis buffer (1% Triton X-100, 0.5% Nonidet-40, 10 mM dithiothreitol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 100 μg/ml bacitracin, 100 μg/ml benzamidine, 2 mM sodium orthovanadate, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM sodium pyrophosphate, 50 mM KH2PO4, 10 mM sodium molybdate and 20 mM Tris-HCl, pH 7.4) at 4°C for 20 minutes. After centrifugation of the cell debris at 14,000 ***g*** for 30 minutes, clarified lysates were subjected to immunoprecipitation for 2 hours with antibodies specific for the β_2AR (CM04) linked covalently to agarose beads. Immune complexes were washed three times with RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM DTT, 1% Triton X-100, pH 8.0) and separated on 10% SDS-acrylamide Laemmli gels. Immunoblotting and detection of the β-tubulin or actin were performed with anti-β-tubulin and anti-actin antibody (both from Sigma), respectively, as previously described (Fan et al., 2001a; Fan et al., 2001b).

Confocal microscopy

For the confocal microscopy studies, cells expressing β_2-AR and grown in eight-well Nunc chamber slides were serum starved and treated as indicated. Objects were imbedded in SlowFade (Molecular Probes) anti-fade reagent. Images were acquired on the Nikon Eclipse E600 microscope (oil-immersion, 60× objective) using He and Ne lasers. The digital images were exported as TIFF files and analyzed in Adobe Photoshop 5.5.

For immunofluorescent staining, cells were fixed with 3% paraformaldehyde and 0.25% glutaraldehyde in PBS pH 7.4 containing 2% of sucrose, permeabilized with 0.1% of Triton X-100 and incubated with blocking buffer (1% of normal goat serum in PBS-Triton X-100) for 1 hour at room temperature. Fixed cells were incubated with primary antibody for 30 minutes (37°C); this was followed by extensive washing and a 30 minute incubation with the Alexa 655 conjugated secondary antibody (Molecular Probes, Eugene, OR) and final wash steps.

Hormone stimulation and drug treatment studies

A431 cells and stably transfected clones were routinely stimulated with either isoproterenol (10 μM) or insulin (100 nM) for 30 minutes and the trafficking of the GFP-tagged receptor monitored by fluorescence microscopy. Cells were serum-deprived for 18 hours before stimulation to remove growth factors and catechols from the cell media. For studies of the role of cytoskeleton elements in receptor trafficking, drugs were added either 30 minutes (for treatment with latrunculin or taxol) or 15 minutes (for treatment with nocodazole) in advance of the challenge with hormones. For studies with nocodazole only, cells were pre-cooled on ice before addition of the drug, to insure that the microtubules were fully depolymerized. The concentrations at which the drugs were used are as follows: nocodazole, 10 μM; taxol, 1 μM; and latrunculin, 1 μM.

β-Adrenergic antagonist binding and receptor sequestration

The expression of β_2ARs in stably transfected A431 and CHO clones was quantified using the radiolabeled, high-affinity β-adrenergic antagonist [^{125}I]iodocyanopindolol (ICYP) to bind to intact cells. Identical radioligand binding assays were performed using the radiolabeled, water-soluble, membrane-impermeant [^{3}H]CGP-12177 to determine the amount of cell-surface β_2AR in the untreated cells, as well as in the cells treated with either insulin or isoproterenol in the absence or presence of the cytoskeletal inhibitors/stabilizers (Karoor and Malbon, 1998). The data are presented as mean±s.e.m., where the amount of cell-surface receptor in the untreated cells is set as 100%.

Results

Human epidermoid carcinoma A431 cells are a popular model for studying β_2AR biology and regulation (Morris and Malbon, 1999). The regulation of the receptor was studied using human β_2AR tagged on the C-terminus with GFP (Gagnon et al., 1998) and examined by confocal microscopy (Fan et al., 2001a; Fan et al., 2001b). A431 cells respond to the β-adrenergic agonist isoproterenol with activation of the β_2AR, accumulation of cyclic AMP, desensitization, internalization, resensitization and recycling of receptor back to the cell surface (Shih and Malbon, 1994). Activation of the insulin receptor tyrosine kinase leads to counterregulation of the β_2AR, uncoupling from Gs and internalization (Karoor and Malbon, 1998). Treatment of A431 cells with insulin (100 nM) or isoproterenol (10 μM) led to a marked internalization of β_2AR (Fig. 1, control). In the absence of either hormone, β_2ARs were localized largely to the cell membrane (yellow arrows throughout), whereas treatment with isoproterenol or insulin leads to internalization of β_2AR (white arrowheads throughout). These changes were quantified by the equilibrium radioligand binding studies, which made use of the water-soluble, cell-impermeant β-adrenergic antagonist ligand [^{3}H]CGP-12177 to quantify the amount of cell-surface β_2ARs.

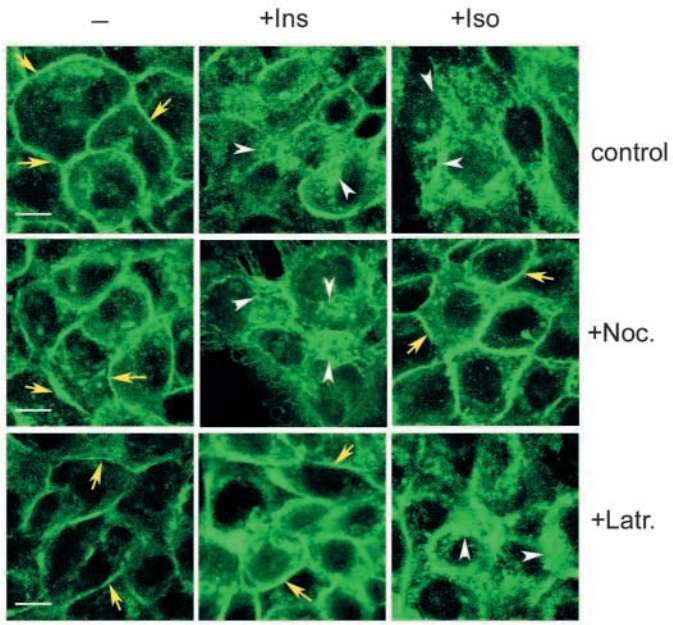


Fig. 1. Distinct elements of cytoskeleton are essential for sequestration of β_2AR induced by stimulation of cells with insulin (+Ins) or isoproterenol (+Iso). A431 cells stably expressing β_2AR tagged with enhanced GFP were used to monitor receptor internalization (by confocal microscopy) following stimulation with β-adrenergic agonist (10 μM isoproterenol) or 100 nM insulin. Control cells, without any pretreatment or cells treated with cytoskeletal disrupting drugs nocodazole (10 μM) or latrunculin (1 μM) were subsequently stimulated with either insulin (100 nM, +Ins) or isoproterenol (10 μM, +Iso) for 30 minutes. After the fixation and embedding in SlowFade, cells were analyzed by confocal microscopy. In the control experiment (top panel), receptor relocated from cell periphery (area of plasma membrane, marked with yellow arrows) to nuclear proximity (white arrowheads). In the presence of nocodazole (+Noc.), stimulation with insulin (+Ins) induced sequestration of receptor from plasma membrane, whereas in cells stimulated with isoproterenol (+Iso), receptor remained on cell periphery (yellow arrows). Pretreatment of cells with latrunculin (+Latr.) suspended β_2AR relocation in response to stimulation with insulin. Bars, 10 μm.

When compared with the percentage of cell-surface localized β_2ARs observed in untreated cells, the amount of β_2ARs lost to internalization in response to isoproterenol was 42±3.4% (n=6) and in response to insulin, 31±4.0% (n=6), as measured by [^{3}H]CGP-12177 binding to intact A431 cells in suspension. This level of internalization agrees well with previous data on the internalization of β_2ARs in response to isoproterenol and to insulin in other cell lines (Karoor et al., 1998; DeCostanzo et al., 2002; Morris and Malbon, 1999).

It has been known for many years that microtubules have a role in the trafficking of GPCRs, including the β_2AR (Limas and Limas, 1983). We examined what effects disruption of the microtubules with nocodazole would have on the trafficking of β_2AR, observing that this agent, which in vivo binds tubulin and depolymerizes microtubules, blocks isoproterenol-induced internalization of β_2ARs (Fig. 1, +Noc.). Remarkably, nocodazole did not influence the response to insulin; insulin stimulated a robust internalization of β_2AR even in the presence of this microtubule inhibitor. Thus, we gained the first insight that the trafficking of the β_2AR by two potent regulators of internalization had some fundamental differences in mechanism. The role of the other major cytoskeletal system, the microfilaments formed from F-actin, was examined using latrunculin A, which binds the actin monomer and blocks F-actin dynamics. Treating A431 cells with latrunculin A had little influence on the sequestration of β_2ARs in response to isoproterenol, but effectively blocked the ability of insulin to internalize the β_2AR (Fig. 1, +Latr.).

The effects of nocodazole were examined by analyzing the trafficking of the GFP-tagged receptor and by confocal analysis of cells stained with anti-β-tubulin (Fig. 2). Microtubules stained prominently in these epidermoid carcinoma cells (Fig. 2A), the patterns of microtubules being somewhat sensitive to treatment of the cells with either 100 nM insulin (+Ins) or with the β-adrenergic agonist isoproterenol (10 μM, +Iso). In untreated cells, the majority of the β_2ARs were localized to the cell membrane. Merging the two images shows the redistribution of microtubules and marked internalization of β_2ARs that occurs when the cells are treated with either insulin (i.e. counterregulation) or isoproterenol (i.e. agonist-induced sequestration). The β-agonist-induced induction of the β_2ARs, by contrast, was not observed in the nocodazole-treated cells (Fig. 2B). Treatment with nocodazole provoked a profound destabilization of the microtubular network in the untreated and hormone-treated cells alike (Fig. 2B). Microtubules were markedly shortened in the nocodazole-treated cells. Remarkably, in spite of the loss of much of the cytoskeletal architecture by depolymerization of microtubules, the internalization of β_2ARs in response to insulin stimulation was essentially the same as noted in the control cells (Fig. 2A). Treating the cells with latrunculin A did not alter the localization of the β_2ARs at the cell membrane in the absence of hormones (Fig. 2C). Agonist-induced sequestration of β_2ARs in response to isoproterenol proceeded normally in cells treated with latrunculin A (Fig. 2C). The ability of insulin to counterregulate β_2ARs and provoke internalization, by contrast, was essentially blocked in the latrunculin A-treated cells.

Taxol stabilizes microtubules by binding to a pocket of β-tubulin on the inner surface of a microtubule. This negates the effects of GTP hydrolysis that drives the depolymerization occurring on the other side of the monomer (He et al., 2001). Taxol treatment of A431 cells had a counterintuitive effect on the trafficking of β_2ARs. Treatment with taxol stabilized the microtubules, while still permitting the internalization of receptor in response to stimulation of either insulin or isoproterenol (Fig. 3A). Internalization of β_2ARs in response to either insulin or isoproterenol was attenuated modestly by taxol. Well-defined, cell-membrane localization of β_2ARs was evident, although perinuclear accumulation of receptor in response to either hormone was also noted. The distribution of receptors appears to be more homogeneously partitioned between the cell membrane and the perinuclear sites of accumulation, with less receptor found elsewhere in the cytoplasmic compartment. The effects of taxol treatment on the microtubules were profound, as noted by epifluorescence analysis of the β-tubulin (Fig. 3B). In taxol-treated cells, relocalization of the microtubular network at the cell membrane was prominent. Stimulating taxol-treated cells with either isoproterenol or insulin reduced the accumulation of

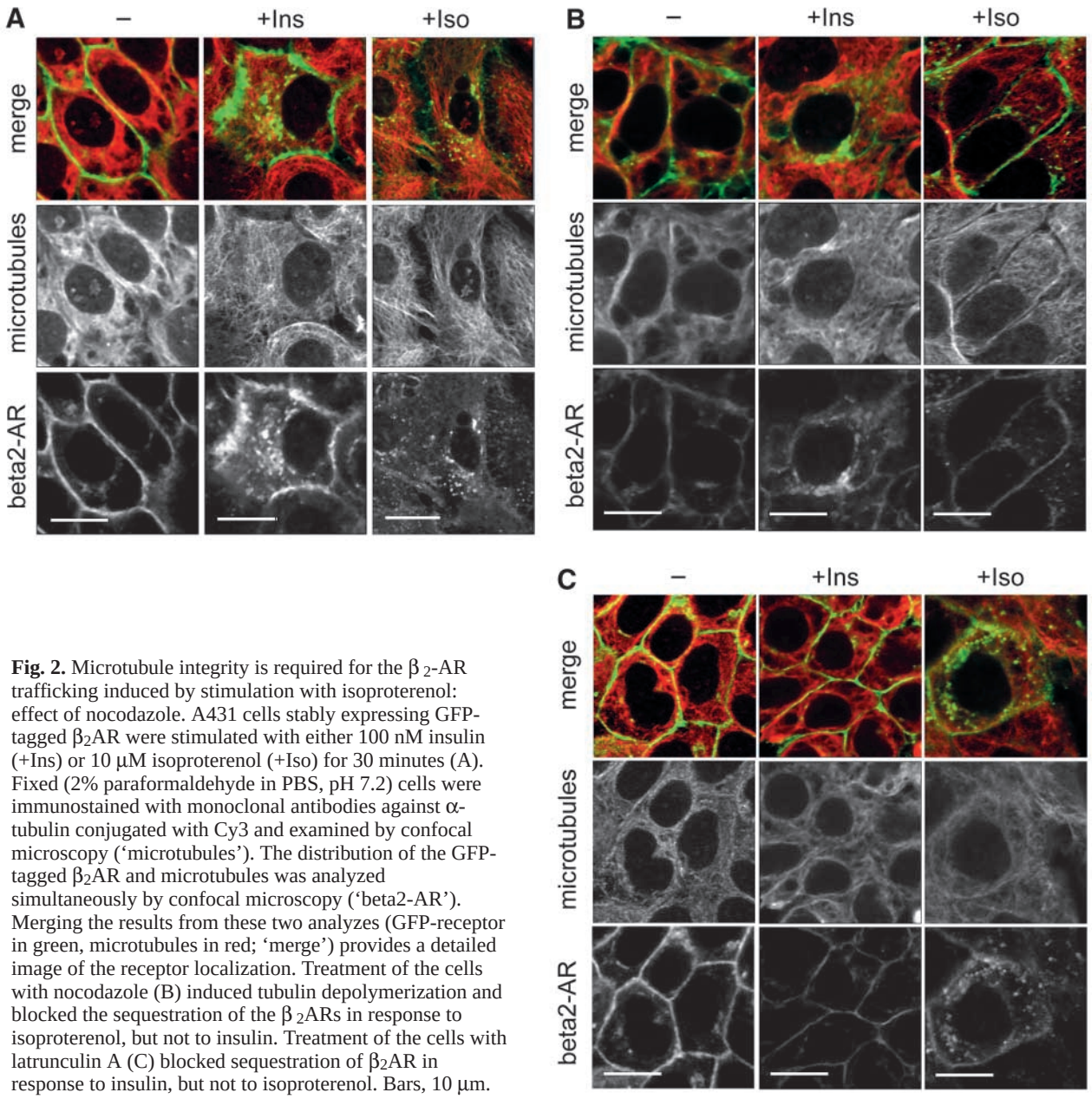


Fig. 2. Microtubule integrity is required for the β_2-AR trafficking induced by stimulation with isoproterenol: effect of nocodazole. A431 cells stably expressing GFP-tagged β_2AR were stimulated with either 100 nM insulin (+Ins) or 10 μM isoproterenol (+Iso) for 30 minutes (A). Fixed (2% paraformaldehyde in PBS, pH 7.2) cells were immunostained with monoclonal antibodies against α-tubulin conjugated with Cy3 and examined by confocal microscopy ('microtubules'). The distribution of the GFP-tagged β_2AR and microtubules was analyzed simultaneously by confocal microscopy ('beta2-AR'). Merging the results from these two analyzes (GFP-receptor in green, microtubules in red; 'merge') provides a detailed image of the receptor localization. Treatment of the cells with nocodazole (B) induced tubulin depolymerization and blocked the sequestration of the β_2ARs in response to isoproterenol, but not to insulin. Treatment of the cells with latrunculin A (C) blocked sequestration of β_2AR in response to insulin, but not to isoproterenol. Bars, 10 μm.

microtubules at the cell membrane and the formation of microtubule arrays elsewhere in the cell (Fig. 3B).

Components of the multivalent, signaling complexes associated with the β_2AR include AKAP250 (gravin), AKAP79, protein kinase A, protein kinase C, Src and protein phosphatase 2B (Shih and Malbon, 1994; Cong et al., 2001; Lin et al., 2000; Fan et al., 2001a; Fan et al., 2001b; Oliveria et al., 2003). β_2AR signaling complexes were isolated by immunoprecipitation to ascertain whether or not actin or β-tubulin could be detected in the complex (Fig. 4). Analysis of the immune precipitations with anti-β_2AR antibodies revealed the presence of both actin and β-tubulin. Treatment with insulin or isoproterenol increased the amount of both cytoskeletal elements associated with the signaling complexes. AKAP250 (gravin), a scaffold protein for the β_2AR, displays an F-actin binding site (Gelman, 2002). Other elements of GPCR signaling complexes have been reported to interact with microtubules (Roychowdhury and Rasenik, 1994; Wang et al., 1990). Immunoprecipitations performed with unrelated antibodies and with unmodified matrix failed to pull down cytoskeletal elements, as determined by staining of immunoblots of the precipitates with either anti-β-tubulin or anti-F-actin antibodies (data not shown).

Compared with what is known about agonist- and insulin-induced internalization of β_2ARs, little is known about the recycling of receptor back to the cell membrane and what role, if any, the cytoskeleton plays in this process. To address the role of the F-actin cytoskeleton and the microtubular network in β_2AR recycling, cells were first stimulated with either insulin or isoproterenol for 30 minutes to induce full receptor sequestration. The cells were then either washed free of insulin to induce recovery from insulin treatment or incubated with 10

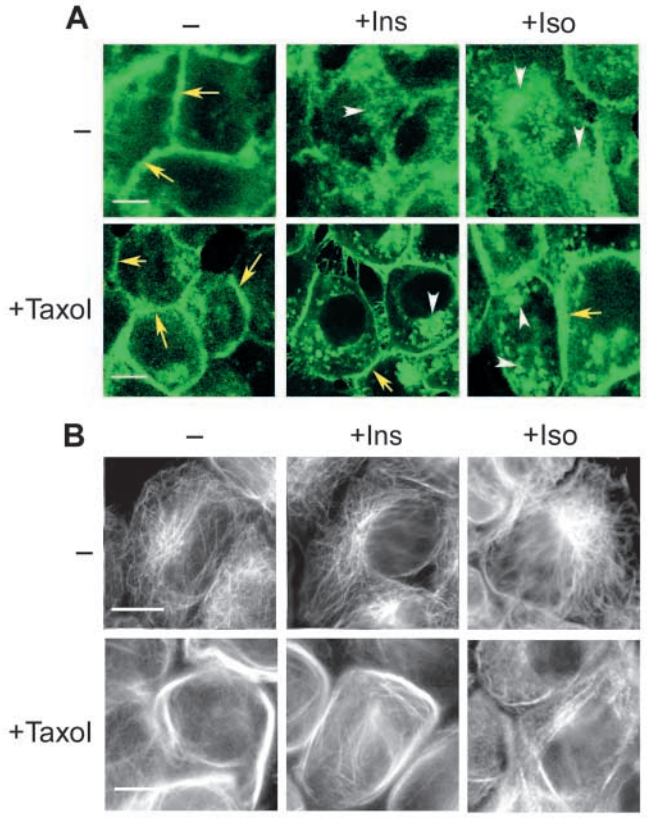


Fig. 3. Taxol-induced polymerization of microtubules does not prevent receptor sequestration in response to stimulation. A431 cells stably expressing GFP-tagged β_2AR (A) or wild-type (B) were stimulated with either insulin or isoproterenol for 30 minutes. Taxol (10 μM, '+Taxol') was added to cells for 30 minutes in advance. (A) GFP-tagged β_2AR internalization following stimulation was fixed and analyzed by confocal microscopy (control, upper panel). Pretreatment with taxol (lower panel) does not significantly affect receptor internalization in stimulated cells. (B) The same experiment performed on wild-type A431 cells: fixed (2% paraformaldehyde in PBS, pH 7.2) wild-type cells were immunostained with monoclonal antibodies against α-tubulin coupled with FITC. Images from confocal microscopy showed the typical pattern of microtubules cytoskeleton in A431 cells (control experiment, upper panel). Treatment with taxol (lower panel) induced its polymerization and redistribution. Polymerized microtubules formed bulky rigid structures, localized in the cell periphery, in a parallel manner to the plasma membrane. Additional stimulation with isoproterenol ('+Iso') counteracts the effects of taxol and restores the arrangement of microtubules, radiating from nuclear vicinity with microtubules forming prominent arrays. Bars, 10 μm. β_2ARs localized either to the cell membrane (yellow arrows) or to the intracellular space (white arrowheads).

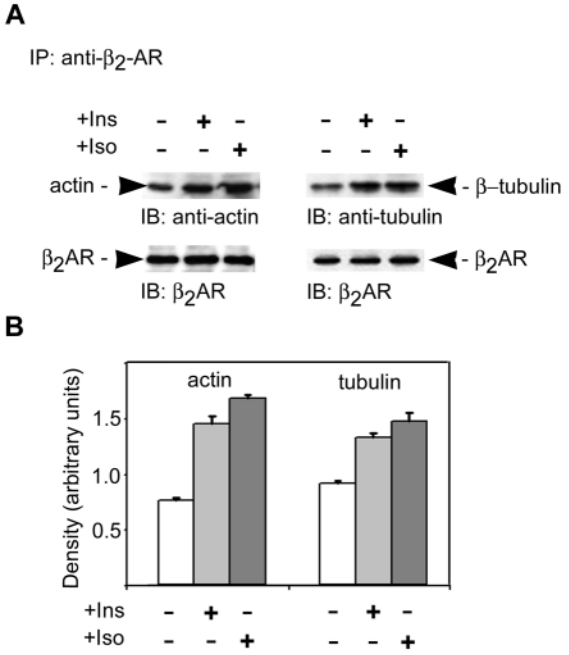


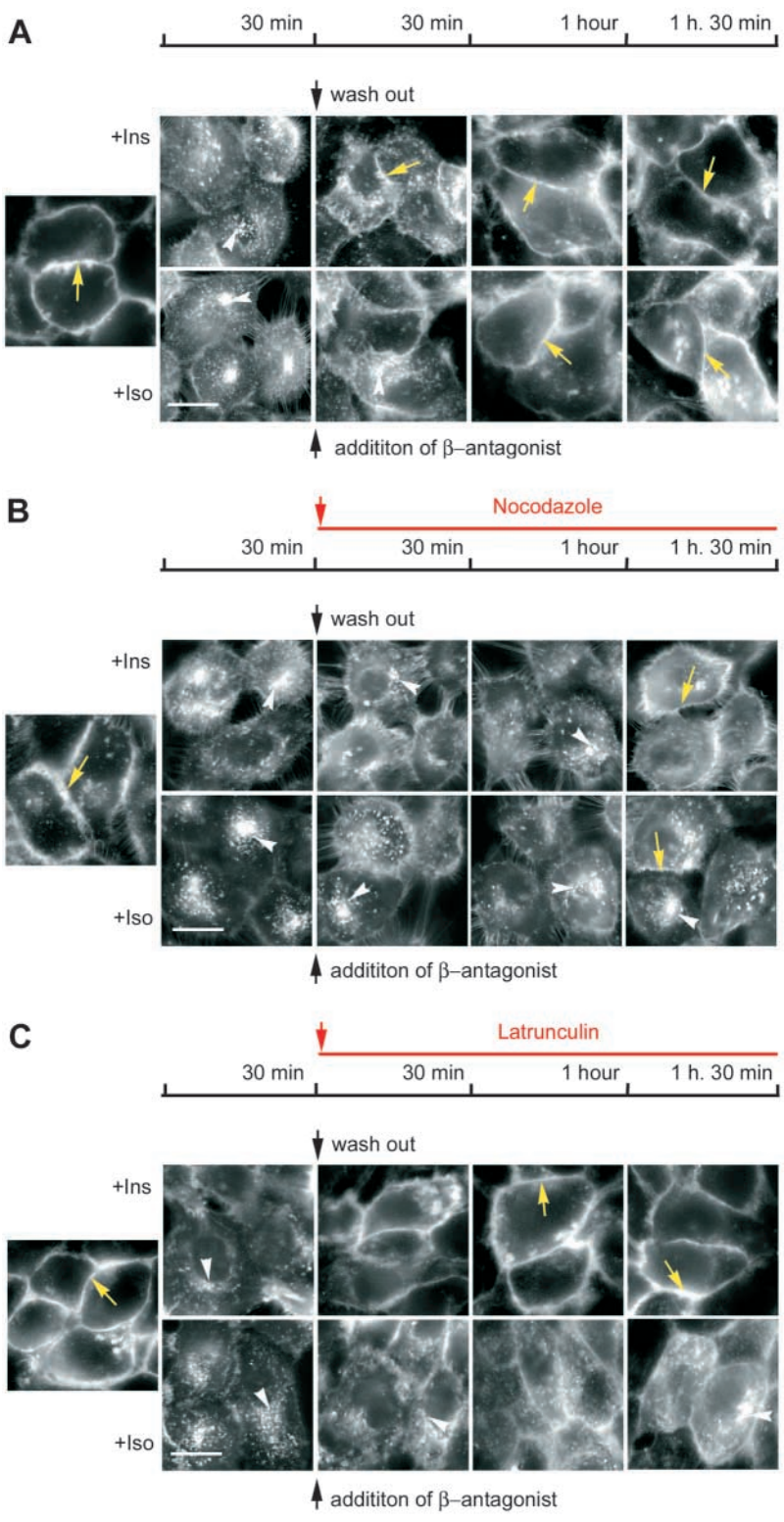
Fig. 4. Stimulation with both insulin and isoproterenol results in an increased association of β_2AR with actin and tubulin. (A) Wild-type A431 cells were stimulated with either insulin or isoproterenol for 30 minutes. Cell lysates were immunoprecipitated using β_2AR-specific antibodies. Immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotted with anti-tubulin, anti-actin or anti-β_2AR antibodies. Western blots show the increased density of bands corresponding to actin and tubulin. Bands, detected by β_2AR-antibodies, confirmed the equal loading of the samples. (B) Quantification of actin and tubulin detection in precipitates: data from western blots (as in A) were quantified by measuring area density (Adobe PhotoShop). The graphs show that the association of β_2AR with actin and tubulin is much greater in stimulated cells than that in unstimulated cells.

μM propranolol – a high-affinity, β-adrenergic antagonist – to block isoproterenol binding to the receptors, and allowed to recover for up to 90 minutes (Fig. 5A). After 30 minutes with either insulin or isoproterenol, the bulk of the receptor was sequestered to perinuclear areas away from the cell membrane. The time-course for recovery reveals a progressive recycling of the β_2ARs to the cell membrane that is largely complete within 60-90 minutes. To test the role of microtubules in the recovery phase and in recycling of β_2ARs, cells were treated with either insulin or isoproterenol for 30 minutes and then co-treated with nocodazole for 30 minutes and finally washed free of insulin (insulin-induced) or treated with propranolol (isoproterenol-induced) in buffer containing nocodazole (Fig. 5B). The disruption of microtubules led to the attenuation of the recycling of receptors from isoproterenol- or insulin-treated cells. Although microtubule dynamics were not important to the ability of insulin to stimulate internalization of the β_2ARs, microtubules appeared essential to the recovery phase and recycling of the receptors back to the cell membrane. For β_2ARs internalized in response to isoproterenol, the recycling also was impaired by nocodazole. Thus, the internalization of β_2ARs in response to β-adrenergic agonist, as well as the recycling of the internalized β_2ARs back to the cell membrane, was blocked by disruption of microtubules.

To test the role of F-actin cytoskeleton in the recovery phase and recycling of β_2ARs, cells were stimulated with either insulin or isoproterenol for 30 minutes and then co-treated with latrunculin A for 30 minutes and finally washed free of insulin (insulin-induced) or treated with propranolol (isoproterenol-induced) in buffer containing latrunculin A (Fig. 5C). Disruption of F-actin dynamics markedly attenuated the recycling of receptors in cells stimulated with isoproterenol. The effects of latrunculin-A treatment on the recycling of

receptors internalized in response to insulin stimulation were, by contrast, more modest. Normal F-actin dynamics were important to the ability of insulin to internalize the β_2ARs, but were nonessential to the recovery phase and recycling of the receptors back to the cell membrane (Fig. 5C). For β_2ARs internalized in response to isoproterenol, the recycling was impaired by latrunculin A, whereas the internalization process in response to β-agonist was essentially insensitive to the disruption of F-actin cytoskeleton.

We noted the ability of isoproterenol to stimulate a dramatic change in the organization of the microtubule network (Fig. 2) and wondered whether the change was a result of the receptor activation per se or the most probable downstream response to the β-adrenergic agonist, i.e. increased accumulation of intracellular cyclic AMP. Treating the cells with the plant diterpene forskolin led to robust accumulation of intracellular cyclic AMP by activating the adenylyl cyclase and bypassing the receptor/G-proteins upstream. Treatment with forskolin alone produced a pattern of microtubules somewhat similar to that observed in response to isoproterenol, suggesting that the regulation is probably dependent on both cyclic AMP and protein kinase A (Fig. 6). Nocodazole disrupted the microtubule network, but one could still observe arrays of fine microtubules stimulated by isoproterenol or by forskolin treatment. The effects of taxol on the stabilization of microtubules were profound (Fig. 3B). Treatment with either isoproterenol or forskolin (and presumably elevated cyclic AMP levels), by contrast, led to the appearance of radiant microtubules that were not present in the cells treated with taxol alone (Fig. 6).



Discussion

Insulin signaling dominates two major cellular events, mitogenesis and metabolic regulation.

Fig. 5. The integrity of the cytoskeleton is required for the recycling of β_2AR back to the plasma membrane. (A) Clones expressing β_2AR tagged GFP were stimulated with 100 nM insulin (+Ins, upper row) or with 10 μM isoproterenol (+Iso, bottom row) for 30 minutes, then removed from stimulation by either washout alone (for insulin) or washout and addition of the high-affinity β-adrenergic antagonist propranolol (10 μM) (for isoproterenol-treated cells). The recovery process was monitored over a time period of 180 minutes, using confocal microscopy. In both cases, 180 minutes after washout the receptors are found to be relocated from the cytoplasm (white arrowheads) back to plasma membrane (yellow arrows). (B) Perturbation of microtubule cytoskeleton with nocodazole prevents the recycling of β_2ARs. Nocodazole was added to cultures after the stimulation by hormones and simultaneously with the termination of stimuli. Subsequent monitoring of receptor recovery revealed that after sequestration in response to stimulation either by insulin or by isoproterenol, recycling of β_2ARs back to the plasma membrane was impaired, i.e. a large pool of GFP-tagged receptor can be observed in the cytoplasm (white arrowheads) rather than in the cell membrane (yellow arrows). (C) Actin depolymerization blocks the recycling of β_2AR after its internalization induced by stimulation with isoproterenol. Clones were treated as above, except with latrunculin, rather than nocodazole. Scale bars: 10 μm. β_2ARs localized either to the cell membrane (yellow arrows) or to the intracellular space (white arrowheads).

These events are focused largely on insulin action at the level of skeletal muscle, liver and adipose tissue, but are observed to some extent in all cells (Olefsky, 1999; Czech and Corvera, 1999; Saltiel and Kahn, 2001). β-Catecholamines generally act to oppose the actions of insulin. Catecholamines stimulate glycogen breakdown, protein degradation, gluconeogenesis and lipolysis, whereas insulin acts to counteract each of these important metabolic pathways. A variety of studies has shown that the ability of insulin to counterregulate the β_2AR is an essential element of insulin action (Morris and Malbon, 1999). Insulin provokes the tyrosine phosphorylation of β_2AR on specific tyrosyl residues (Y350, 354 and 364), which are confined to the cytoplasmic, C-terminal tail of the receptor (Doronin et al., 2000; Karoor et al., 1995). Phosphorylation of the Y350 residue of the β_2AR creates a docking site for SH2 domains of a variety of proteins, including Grb2 and dynamin, and the regulatory subunit of PI3 kinase (Baltensperger et al., 1996). The integrity of Y350 and its phosphorylation in response to insulin are essential for insulin to express its counterregulatory control of β_2AR functions and β_2AR sequestration (Shih and Malbon, 1998).

Our current work provides several new insights into the manner in which β-adrenergic agonists differ from insulin in the trafficking of β_2ARs. Most notably, this study reveals differing roles of the microtubule versus F-actin networks in enabling the internalization of β_2ARs for agonist-induced regulation versus counterregulation by insulin. Agonist-induced internalization of β_2ARs follows desensitization and is a hallmark for virtually all GPCRs (Morris and Malbon, 1999). Agonist-induced sequestration of the β_2AR to perinuclear locales occurs within minutes, but requires 15-30 minutes to reach maximal internalization. Nocodazole in vivo binds tubulin monomer and induces the depolymerization of microtubules. We found that nocodazole effectively blocks internalization of β_2ARs in response to β-agonist, but it had no influence on the ability of insulin to counterregulate β_2ARs through sequestration. Thus, we speculate that an intact microtubular network enables agonist-induced sequestration of GPCRs.

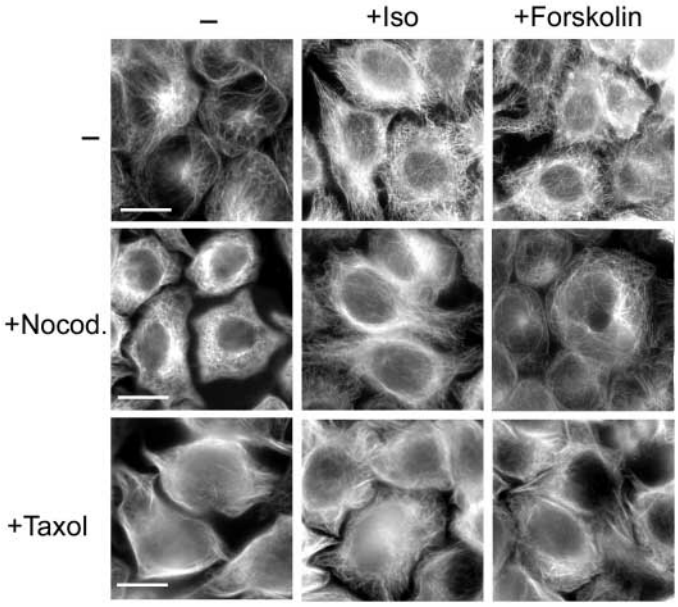


Fig. 6. Stimulation of cells with either isoproterenol or forskolin provokes microtubule reorganization. Untreated A431 cells (control, –), or cells pretreated with either nocodazole or taxol (see legends to Figs 1 and 3) were stimulated with either isoproterenol (10 μM, +Iso) for 30 minutes or with the plant diterpene forskolin (100 μM, +Forskolin) for 15 minutes. After the stimulation, the cells were washed, fixed and immunostained to visualize microtubules (see legend to Fig. 3). Stimulation by isoproterenol induces changes in the organization of microtubules, i.e. the radiance of microtubules from the central regions to the periphery of the cell was more prominent and multiple nucleation centers (note in the forskolin-treated cells) appeared. Treatment either with nocodazole or taxol perturbs these patterns of microtubule organization, i.e. radiating microtubule arrays were absent. Scale bar, 10 μm.

The F-actin cytoskeletal network is essential for many cellular functions. Latrunculin A binds the actin-monomer and acts to sequester actin, blocking F-actin dynamics. In the current study, treatment with latrunculin A showed no influence on agonist-induced internalization of β_2ARs, but rather was found to block insulin-induced sequestration of the β_2ARs. These effects of disrupting actin cytoskeleton on insulin action are not a reflection of an effect proximal to receptor, as insulin receptor autophosphorylation, tyrosine phosphorylation of IRS-1,2 and Cbl, and serine/threonine phosphorylation of Akt in response to insulin are unaffected by latrunculin (Kanzaki and Pessin, 2001). Actin microfilaments do enable the translocation of the GLUT4 glucose transporter to the cell surface in response to insulin, a process that has many similarities to the counterregulation of β_2ARs in response to insulin that operates in the reverse orientation (Shumay et al., 2002). Taken together, these results reinforce the notion that insulin-induced internalization and insulin-induced export of GLUT4 to the cell membrane may constitute use of the same cellular network.

One neglected feature of the agonist-induced trafficking of GPCRs is how the internalized receptors recycle to the cell membrane. We explored whether the recovery from desensitization and from counterregulation by insulin in cells made use of different cytoskeletal elements to recycle the β_2ARs. β_2ARs internalized by either β-agonist or insulin displayed similar time-courses for the recycling of receptor back to the cell membrane. Although insulin-stimulated internalization of β_2ARs was insensitive to the disruption of the microtubules, the recovery of β_2ARs to the cell membrane was effectively blocked by nocodazole. Surprisingly, although the internalization of β_2ARs by agonist was blocked by nocodazole, the recycling of β_2ARs back to the cell membrane was only partially influenced by treatment with nocodazole. Treatment with latrunculin, by contrast, was effective at blocking the recycling of the β_2ARs internalized in response to β-agonist. Thus, counterregulation by insulin and agonist-induced internalization both provoke a massive sequestration of these GPCRs from the cell surface to perinuclear locales in cells, but make use of very different cytoskeletal elements both to internalize and to recycle the receptors back to the cell surface.

Analysis of microtubules and actin microfilaments in cells challenged with β-adrenergic agonist or insulin indicated that some level of rearrangement occurs in response to stimulated cells with these agents. For β-agonist, it seems that the elevation of cyclic AMP may be responsible for the changes in

microtubule architecture. For the insulin-stimulated cells, the response may be more difficult to define, because the downstream signaling for insulin, unlike β-adrenergic agonist (Morris and Malbon, 1999), is populated with the mitogen-activated protein kinase cascades and many protein kinases and phosphatases whose activities are regulated by insulin (Pessin and Saltiel, 2000; Czech and Corvera, 1999; Saltiel and Kahn, 2001). The β_2AR is a member of a multivalent signaling complex composed of the receptor in combination with AKAP250 (gravin), protein kinases A and C, Src and protein phosphatase 2B, and perhaps transiently with other signaling elements such as the heterotrimeric G-protein Gs. It has been shown that AKAP250 possesses an F-actin binding motif (Gelman, 2002) and that microtubules can bind to Gs (Wang et al., 1990). As tantalizing as these speculations may be, much work will be required to elucidate the molecular details and partners involved in the trafficking of GPCRs in response to agonist, as well as to receptor tyrosine kinases.

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