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β-Adrenergic Receptor Stimulation Promotes $G\alpha_s$ Internalization Through Lipid Rafts: A Study in Living Cells

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Running Title: Agonist Induced Internalization of $G\alpha_s$ from Lipid Rafts

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Abbreviations.

The abbreviations used are GPCR, G protein coupled receptor; Gαs, α subunit of the stimulatory G protein; GFP, green fluorescent protein; βAR, β-adrenergic receptor; GTP, guanosine triphosphate; ISO, isoproterenol; EEA1, early endosome antigen 1 protein; LAMP-1, lysosome associated membrane protein 1; CD, methyl-β-cyclodextrin..

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ABSTRACT

Upon binding hormones or drugs, many G protein coupled receptors are internalized leading to receptor recycling, receptor desensitization and down-regulation. Much less understood is whether heterotrimeric G proteins also undergo agonist induced endocytosis. To investigate the intracellular trafficking of Gαs, we developed a functional Gαs-GFP fusion protein which can be visualized in living cells during signal transduction. C6 and MCF-7 cells expressing Gαs-GFP were treated with 10μM isoproterenol, and trafficking was assessed with fluorescence microscopy. Upon isoproterenol stimulation, Gαs-GFP was removed from the plasma membrane and internalized into vesicles. Vesicles containing Gαs-GFP did not colocalize with markers for early endosomes or late endosomes/lysosomes, revealing that Gαs does not traffic through common endocytic pathways. Furthermore, Gαs-GFP did not colocalize with internalized β_2-adrenergic receptors, suggesting that Gαs and receptor are removed from the plasma membrane by distinct endocytic pathways. Nonetheless, activated Gαs-GFP did colocalize in vesicles labeled with fluorescent cholera toxin B, a lipid raft marker. Agonist significantly increased Gαs protein in Triton X-100 insoluble membrane fractions, suggesting that Gαs moves into lipid rafts/caveolae after activation. Disruption of rafts/caveolae by treatment with cyclodextrin prevented agonist induced internalization of Gαs-GFP as did overexpression of a dominant negative dynamin. Taken together, these results suggest that receptor activated Gαs moves into lipid rafts and is internalized from these membrane microdomains. It is suggested that agonist induced internalization of Gαs plays a specific role in GPCR mediated signaling, and could enable Gαs to traffic into the cellular interior to regulate effectors at multiple cellular sites.

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G protein-coupled receptors (GPCRs) are the largest family of signaling molecules in the human genome. They couple to a diverse family of heterotrimeric G proteins that transduce chemical and sensory signals from the receptor to a variety of effectors, such as second messenger generating enzymes and ion channels. With respect to many GPCRs, agonist activation of receptors initiates processes in the cell which lead to receptor desensitization and internalization of the receptors by endocytosis. β-adrenergic receptors (βARs) are prototypic GPCRs that have been studied in detail, particularly with respect to their agonist induced internalization (Claing et al., 2002). Upon agonist binding, the majority of GPCRs are trafficked into clathrin coated pits and internalized by endocytosis (Claing et al., 2002; von Zastrow, 2003). However, some receptors appear to be preferentially located and internalized through specialized lipid raft/caveolae microdomains of the plasma membrane (Claing et al., 2002; Nabi and Le, 2003), a process known as clathrin-independent endocytosis. The GTP binding protein dynamin plays an essential role in both types of receptor endocytosis by acting to liberate endocytic vesicles from the plasma membrane (Nichols, 2003). Lipid rafts and caveolae are plasma membrane microdomains enriched in cholesterol and glycolipids making them highly hydrophobic and insoluble to non-ionic detergents such as Triton X-100. Several signaling proteins including receptors, G proteins and effectors are enriched in both rafts and caveolae, suggesting that these microdomains are involved in G protein mediated signaling. Previous investigations have demonstrated that Gαs is in fact targeted to and enriched in lipid rafts (Oh and Schnitzer, 2001). While agonist induced endocytosis of GPCRs is well characterized, relatively few studies have examined internalization of heterotrimeric G proteins.

Gαs is localized primarily at the plasma membrane, where it allosterically activates its classic effector, adenylyl cyclase, resulting in the production of cAMP during receptor signaling

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events. It has become increasingly clear that Gαs is also located in other cellular compartments. Gαs has been detected in endocytic vesicles obtained from liver (Van Dyke, 2004), it associates with tubulin and the microtubule cytoskeleton in neuronal cells (Roychowdhury et al., 1999; Sarma et al., 2003), and Gαs is enriched in the trans Golgi network of rat pancreatic cells (Denker et al., 1996). Several studies have indicated other functional roles for Gαs apart from activation of adenylyl cyclase, including regulation of apical transport in liver epithelia (Pimplikar and Simons, 1993), regulation of endosome fusions (Colombo et al., 1994) and controlling the trafficking and degradation of epidermal growth factor (EGF) receptors (Zheng et al., 2004). How Gαs is trafficked to these cellular locations and the mechanism governing its association with these subcellular compartments remains unclear.

Several previous studies have indicated that Gαs undergoes a redistribution from the plasma membrane to cytosol in response to agonist stimulation (Hynes et al., 2004b; Ransnas et al., 1989; Thiyagarajan et al., 2002; Wedegaertner and Bourne, 1994; Wedegaertner et al., 1996; Yu and Rasenick, 2002), however there are reports that have failed to see this redistribution (Huang et al., 1999; Jones et al., 1997). Recently, a fluorescent Gαs-GFP fusion protein was developed by inserting green fluorescent protein into the internal sequence of Gαs. This Gαs-GFP fusion protein binds GTP in response to agonist, activates adenylyl cyclase, is appropriately expressed at the plasma membrane and exhibits identical trafficking and signaling behavior as the wild type Gαs (Yu and Rasenick, 2002). During βAR stimulation, activated Gαs-GFP rapidly dissociates from the plasma membrane in living cells (Yu and Rasenick, 2002). The mechanism controlling the release of Gαs from the membrane is not yet known, but it has been suggested that activated Gαs is depalmitoylated and then released from the membrane

(Wedegaertner and Bourne, 1994). The ultimate redistribution and putative signaling of internalized Gαs is poorly understood.

We have hypothesized that similar to receptor, Gαs internalizes in response to agonist and associates with endocytic vesicles. Using real-time imaging of Gαs-GFP during agonist stimulation, this report demonstrates that Gαs dissociates from the plasma membrane and becomes internalized in vesicles. Internalized Gαs-GFP containing vesicles were derived from lipid raft domains, but were not common to early or late endosomes. In addition, internalized Gαs-GFP did not colocalize with β_2ARs in vesicles, suggesting that receptor and Gαs traffic through distinct endocytic pathways. It is suggested that agonist induced internalization of activated Gαs may regulate endocytic trafficking and play a specific role in GPCR mediated signaling, and could enable Gαs to traffic into the cellular interior to interact with effectors at multiple cellular sites.

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MATERIALS AND METHODS

Cell Culture and Transfections. MCF-7 human breast adenocarcinoma and C6 rat glioma cell lines, both of which express endogenous β_2ARs, were used for these experiments. MCF-7 cells were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum, 1% penicillin and streptomycin and maintained in 5% CO_2 at 37ºC. C6 cells were cultured in DMEM containing 4.5 g glucose/L, 10% calf serum supplemented with iron (Hyclone), 1% penicillin and streptomycin and maintained in 10% CO_2 at 37ºC. Details explaining construction of the Gαs-GFP fusion protein has been described previously (Yu and Rasenick, 2002). The cDNA encoding the dominant negative K44E dynamin 1 was originally obtained from Dr. Richard Vallee (Columbia, NYC) (Herskovits et al., 1993), and it was subsequently cloned into pcDNA3.1zeo and kindly provided by Dr. Mark von Zastrow (UCSF, CA) (Chu et al., 1997). Both MCF-7 and C6 cells were seeded into delta T vision 35 mm dishes (Fisher) for live cell imaging or onto coverslips in 12 well plates for immunofluorescence. Cells were grown to 80% confluency and were transfected for 5 hours with 0.5 µg of purified Gαs-GFP plasmid DNA per dish or well, using a ratio of 1:5, DNA : superfect transfection reagent (Qiagen). 24 hours after Gαs-GFP transfection, cells were used for imaging experiments. Gαs-GFP expression in both MCF-7 and C6 cells was semi-quantified by western blotting. Gαs-GFP expression was approximately 3 fold higher than endogenous Gαs expression. Co-expression of Gβγ was not required for proper membrane association of Gαs-GFP. Thus, presumably, Gαs-GFP utilizes the endogenous Gβγ for this purpose. For the dominant negative dynamin 1 experiments, C6 cells were co-transfected for 5 hours with 0.5 µg of Gαs-GFP and 1.0 µg of K44E dynamin plasmid DNA per dish, using a ratio of 1:5, DNA : superfect transfection reagent. 16 hours after the co-transfections, cells were used for imaging experiments.

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Live Cell Imaging and Immunofluorescence Microscopy. 1 hour prior to live cell imaging, complete media was replaced with serum-free DMEM supplemented with 20 mM HEPES. Cells were maintained at 37ºC during the entire period of observation using a heated microscope stage (Biotechnics, Fisher). Fluorescent images were obtained using an inverted microscope equipped for fluorescent microscopy (Nikon Eclipse TE 300, 547 nm wavelength excitation, 579 nm emission, via high pressure Nikon Xenon XBO 100 W lamp); a digital camera (RTE/CCD-1300 Y/HS, Roper Scientific; MicroMAX camera controller, Princeton Instruments Inc.; Lambda 10-2 shutter, Sutter Instruments Co.), and image-processing software (IPLab, Scanalytics Inc.). All images shown were obtained using oil immersion with a 60x objective. Scale bars shown are 10 micrometers in length. Cells were treated with 10μM isoproterenol (Sigma) and Gαs-GFP trafficking was imaged in real time during receptor stimulation. For live cell imaging using transferrin Texas red ligand or fluorescent cholera toxin B-Alexa 555 (Molecular Probes), MCF-7 cells expressing Gαs-GFP were pre-incubated with the probes for 20 minutes on ice (10 μg/ml transferrin, 400ng/ml cholera toxin B). Cells were then washed and immediately warmed to 37ºC in the presence of 10μM isoproterenol during imaging. For imaging studies utilizing the cholesterol chelating agent methyl-β-cylcodextrin (CD) (Sigma), C6 cells expressing Gαs-GFP were pre-incubated with 10mM cyclodextrin for 30 min. at 37ºC, cells were washed, and subesequently imaged during treatment with 10μM isoproterenol. To reverse the effects of CD, cholesterol was added back to cells that were initially incubated with CD. These cells were treated for 30 min. with CD, washed with DMEM, and then treated with CD-cholesterol complexes for 90 minutes (10 μg/ml cholesterol-CD in a molar ratio of 1:6, Sigma cat# C4951) to deliver cholesterol back to the cells (Ostrom et al., 2004). These recovered cells were washed, and subesequently imaged during treatment with

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10μM isoproterenol. For immunofluorescence microscopy, Gαs-GFP transfected MCF-7 cells were treated with 10μM isoproterenol and were then fixed with 3.3% paraformaldehyde. Cells were permeabilized and blocked for 1h with 0.5% saponin, 5% BSA, 1x PBS. Cells were incubated with the following primary antibodies for 3 hours: rabbit polyclonal anti EEA1 antibody (1ug/ml dilution, BD Biosciences), mouse monoclonal anti LAMP1 (1 ug/ml dilution, Univ. of Iowa Developmental Hybridoma Bank) or with rabbit polyclonal anti B_2AR antibody with overnight incubation (1:100 dilution, Santa Cruz Biotech.) Cells were incubated with secondary antibodies for 1 h: goat anti mouse IgG rhodamine (1:100 dilution, Pierce) or goat anti-rabbit IgG rhodamine (1:100 dilution, Boehringer Mannheim). Coverslips were mounted and cells were imaged using fluorescence microscopy as described above. Images of live and fixed cells shown are representative of 40 to 50 cells imaged in four or more separate experiments.

Quantification of Gαs-GFP Internalization. Quantification of the internalization of Gαs-GFP was done as previously described (Yu and Rasenick, 2002). An individual blind to the experimental conditions performed all measurements. The mean of gray value within the cytoplasm in fluorescence images were collected by selecting an area that corresponded to the maximal cytoplasmic region for each cell using Scion Image from the Public Domain NIH Image Program. Mean gray values of the Gαs-GFP fluorescence in the cytoplasm were obtained and normalized per area measured. Variation of mean gray values in cytoplasm represents the change of Gαs-GFP fluorescence in the interior of the cell.

Subcellular Fractionation. Confluent C6 cells in 25 cm^2 flasks were treated as described in figure legends. After treatment, cells were harvested into 1 ml of PBS containing 1x protease inhibitors (Roche Diagnostics, complete protease inhibitor cocktail

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cat#11697498001) and homogenized with 10 strokes of a Potter-Elvehjem homogenizer, nuclei were removed by centrifugation at 1000 x g for 10 minutes, and total cellular membranes and purfied cytosol were obtained by 200,000 x g centrifugation for 1 hour using a TLA-45 rotor and Beckman TL-100 table top ultracentrifuge. 10 ug samples of membrane pellet and cytosol (soluble fractions) were analyzed for Gαs content by immunoblotting as described below.

Isolation of Lipid Rafts/Caveolae C6 cells were used to prepare Triton-insoluble, caveolin enriched membrane fractions by the procedure of Toki et al (Toki et al., 1999), with slight modification. C6 glioma cells were grown in 150-cm^2 flasks until confluent, and incubated in serum-free DMEM for 1 hour prior to all treatments. Cells were treated with 10μM isoproterenol for 10, 30 and 60 minutes. Some cells were treated with 10mM CD for 30 minutes to disrupt lipid rafts/caveloae, or with CD followed by treatment with CD-cholesterol complexes for 90 minutes to re-deliver cholesterol to the cells (as described above under Live Cell Imaging). Two flasks of cells for each treatment group were harvested into 1.0 ml of HEPES buffer (10mM HEPES, pH 7.5, 150mM NaCl, 1mM DTT, 0.3mM PMSF) containing 1x protease inhibitor cocktail (Roche Diagnostics). Cells were homogenized with 10 strokes of a Potter-Elvehjem homogenizer, nuclei were removed by centrifugation at 1000 x g for 10 minutes, and total cellular membranes were obtained from the supernatant by 100,000 x g ultracentrifugation. The total membrane pellet was resuspended in HEPES buffer containing 1% Triton X-100 and incubated on ice for 30 minutes. The homogenate was adjusted to 40% sucrose by addition of an equal volume of 80% sucrose prepared in HEPES buffer, and placed at the bottom of an ultracentrifuge tube. A step gradient containing 30, 15 and 5% sucrose was formed above the homogenate and centrifuged at 200,000 x g in a SW55 rotor for 18 hours. Two or three opaque bands containing the Triton X-100 insoluble floating rafts were confined between the 15 and

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30% sucrose layers. These bands were removed from the gradients, diluted threefold with HEPES buffer and pelleted in a microcentrifuge at 16,000 x g to obtain caveolin enriched samples of lipid rafts/caveolae. To obtain samples of the non-buoyant Triton X-100 soluble membranes, 500µl was removed from the bottom of each ultracentrifuge tube in the 40% sucrose layer (non-buoyant fraction). These samples were precipitated with 1mM trichloroacetic acid in HEPES buffer for 30 minutes on ice, followed by pelleting in a microcentrifuge. These samples of non-raft Triton X-100 soluble membrane protein and the Triton X-100 insoluble lipid rafts/caveolae were subsequently analyzed by immunoblotting.

Immunoblotting. 5µg of each Triton X-100 soluble membrane fraction and lipid raft/caveolae fraction was subjected to SDS-PAGE. 10µg of each membrane pellet and cytosol sample was also separated by SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes which were analyzed by western blotting. The PVDF membrane was blocked for 1 h with a Tris-buffered-saline/Tween 20 solution (10mM Tris-HCl, 159mM NaCl, and 0.1% Tween 20, pH 7.4) containing 5% dehydrated milk proteins. After 3 washes with Tris-buffered saline/Tween 20, membranes were incubated with polyclonal rabbit Gαs antibody (1:10,000 dilution, 3 h, Dupont/NEN) or polyclonal B_2AR antibody with overnight incubation (1:200 dilution, Santa Cruz Biotech.). Detection of bound antibody on the blot was assessed with a horseradish peroxidase (HRP)-conjugated, goat anti-rabbit IgG antibody (Jackson Laboratories) visualized by enhanced chemiluminescent detection (ECL, Amersham), and quantitated, after scanning densitometry using Imagequant software (Molecular Dynamics). Both the long (52kDa) and short (45kDa) forms of Gαs were quantified together. Immunodetected Gαs, β_2AR and caveolin-1 bands were quantified and the integrated optical density (IOD) of each band was determined and is expressed as percent control. For some

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experiments, the original membranes were stripped with an acidic glycine buffer (100mM glycine, pH 2.4) and reprobed using a monoclonal mouse anti caveolin-1 antibody (1:1000 dilution overnight, Transduction Labs) followed by immunodetection. To adjust for protein loading errors, amounts of Gαs (both long and short isoforms) in the Triton X-100 insoluble lipid rafts/caveolae were normalized for the level of caveolin-1, and are expressed as Gαs in the caveolin rich fraction.

Statistical Analysis. All quantitated data was analyzed for statistical significance using a one way ANOVA followed by Student-Newman-Keuls multiple comparison test using Prism 3.0, software package for statistical data analysis (Graph Pad Software Inc.). Differences were considered significant at $p<0.05$.

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RESULTS

Real-Time Imaging of Gαs-GFP during β-Adrenergic Receptor Stimulation.

C6 rat glioma and MCF-7 human breast adenocarcinoma epithelial cells are useful cell models for these studies, as both cell types can be easily transfected and they express endogenous β_2ARs which couple to Gαs (Manier et al., 1992; Vandewalle et al., 1990). Both cell lines were transiently transfected with Gαs-GFP. 24 hours after transfection, cells were exposed to the βAR agonist isoproterenol, and Gαs-GFP trafficking was imaged in living cells during receptor stimulation. Prior to agonist treatment, Gαs-GFP localized predominantly at the plasma membrane in C6 cells (Fig. 1A), but within 10 minutes after isoproterenol addition, many punctate vesicular structures appeared throughout the cytoplasm. During 10 minutes of treatment of both cell types, there was a marked decrease in Gαs-GFP membrane localization (Fig 1A and 1B), and a contemporaneous appearance of Gαs-GFP subjacent to the plasma membrane (see supplemental online video 1).

Video 1 of a representative MCF-7 cell shows that Gαs-GFP at membrane extensions rapidly re-organizes to form vesicles containing this protein, and these vesicles traffic to the cell interior, indicating active endocytosis of Gαs-GFP. It is noteworthy that agonist-induced removal of Gαs-GFP from the plasma membrane occurred at some regions of the membrane, but not all. In C6 cells, cellular extensions of membrane enriched in Gαs-GFP were repeatedly observed prior to agonist treatment, consistent with endogenous Gαs localization in these cells (Donati et al., 2001) . During receptor stimulation Gαs-GFP was removed from these structures, suggesting internalization may occur in selective regions of the plasma membrane. Taken together, data show that during agonist stimulation of endogenous β_2ARs, activated Gαs-GFP is removed from the plasma membrane, internalized by endocytosis, and localized in vesicles.

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Dominant Negative Dynamin Inhibits Agonist Induced Internalization of Gαs-GFP.

The GTP binding protein dynamin 1 functions enzymatically to liberate vesicles from the plasma membrane during both clathrin mediated and raft/caveolae mediated endocytosis (Nichols, 2003). The K44E dominant negative dynamin 1 is deficient in GTPase activity rendering it non-functional for vesicle formation (Herskovits et al., 1993). To determine whether Gαs internalization is dynamin dependent, C6 cells were co-transfected with Gαs-GFP and K44E dominant negative dynamin 1 constructs and living cells were imaged during isoproterenol stimulation. Figure 2 reveals that Gαs-GFP is expressed predominantly at the plasma membrane of co-transfected cells prior to βAR stimulation. During 25 minutes of isoproterenol exposure, Gαs-GFP remained at the plasma membrane and did not internalize within vesicles or label puncta in the cellular interior. This suggests that agonist induced internalization of Gαs-GFP is dynamin dependent.

Analysis of Internalized Gαs-GFP Trafficking in the Common Compartments of the Endocytic Pathway.

To determine the identity and trafficking of the vesicles containing internalized Gαs-GFP, antibodies against proteins commonly used as markers for early endosomes and late endosomes/lysosomes were utilized. MCF-7 cells expressing Gαs-GFP were treated for 30 minutes with isoproterenol. Cells were fixed and processed for immunocytochemistry using antibodies against the early endosome antigen 1 protein (EEA1) or the lysosome associated membrane protein (LAMP-1), to label early endosomes or late endosomes/lysosomes, respectively. As previously observed, isoproterenol treatment resulted in internalization of Gαs-

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GFP. Fixed cells showed Gαs-GFP in both vesicles and in the cytoplasm. Prior to agonist exposure, EEA-1 and LAMP-1 were localized to endocytic vesicles and showed a punctate localization within the cell interior. After isoproterenol treatments, internalized Gαs-GFP did not colocalize with EEA1 or LAMP1 proteins in merged images (Fig 3A and 3B). In addition, a time course of agonist treatment was performed between 5 minutes and 1 hour, and none of the time points within this time course showed a measurable colocalization between Gαs-GFP and EEA1 or LAMP-1 (data not shown). Lack of colocalization between Gαs-GFP and EEA-1 or LAMP-1 suggests that Gαs-GFP does not traffic through common endocytic compartments involving early or late endosomes or lysosomes.

Upon agonist binding, β_2ARs are rapidly internalized by endocytosis into clathrin coated pits and they traffic into recycling endosomes (Claing et al., 2002). Transferrin receptors also undergo agonist induced endocytosis into recycling endosomes, and the transferrin receptor and its ligand are commonly used as a marker for these endosomes. To examine if Gαs-GFP internalized into recycling endosomes, colocalization of fluorescent transferrin with Gαs-GFP was examined in living cells during receptor stimulation. Prior to agonist exposure at 4º C, both Gαs-GFP and transferrin texas red were localized at the plasma membrane. 15 minutes after exposure to isoproterenol, many vesicles contained Gαs-GFP, but these vesicles did not colocalize with internalized transferrin (Fig. 3C, merge), indicating that Gαs-GFP does not traffic into recycling endosomes.

Imaging of Internalized Gαs-GFP and β_2AR after Receptor Stimulation.

Since the βARs that couple to and activate Gαs are rapidly internalized after agonist binding, a primary question is whether Gαs accompanies the receptor during endocytosis. To

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test this, Gαs-GFP transfected MCF-7 cells were treated with isoproterenol over a time course and cells were then fixed and incubated with antibody for the β_2AR. It is noteworthy that MCF-7 cells express the B_2AR subtype, which mediate isoproterenol activation of Gαs (Draoui et al., 1991; Vandewalle et al., 1990). Isoproterenol treatment resulted in internalization of both Gαs-GFP and β_2AR and numerous vesicles contained these proteins (Fig 3D). Gαs-GFP was also found in the cytoplasm in the paraformaldehyde fixed cells, similar to the previous results (Fig 3A, 3B). Agonist evoked a slight overlap of Gαs-GFP and β_2AR in internalized vesicles, but no obvious colocalization was observed (Fig. 3D, merge). Cells treated with agonist over a time course from 5 minutes to 45 minutes were similarly examined for Gαs-GFP and β_2AR colocalization, but no clear colocalization could be found at any of these time points (data not shown). This lack of colocalization of Gαs-GFP and β_2AR suggests that receptor and Gαs are internalized by distinct pathways.

Isoproterenol Promotes Gαs-GFP and Cholera Toxin B Colocalization in Vesicles.

Since Gαs-GFP was not found in common endocytic compartments such as early endosomes, recycling endosomes and late endosomes, it was hypothesized that non-clathrin mediated endocytosis may be involved in Gαs-GFP internalization. To test the idea that lipid raft/caveolae mediated endocytosis was involved, the lipid raft marker cholera toxin B was utilized to label lipid rafts in living cells. Fluorescent cholera toxin B is a common marker used to investigate the trafficking of proteins during raft mediated endocytosis in living cells (Nichols et al., 2001; van Deurs et al., 2003). It is noteworthy that it is the A subunit of cholera toxin which binds to and ADP-ribosylates Gαs while the B subunit acts as a ligand and binds to the lipid raft localized ganglioside GM-1. Cholera toxin B is constitutively incorporated into cells

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through lipid raft/caveolae mediated endocytosis (Orlandi and Fishman, 1998). Gαs-GFP transfected MCF-7 cells pre-labeled with fluorescent cholera toxin B, were subsequently treated with agonist, and living cells were visualized using digital fluorescence microscopy. Prior to agonist exposure, Gαs-GFP strongly co-localized with cholera toxin B at the plasma membrane, presumably in lipid raft microdomains (Fig 4, upper panel, merge). Fifteen minutes of agonist exposure resulted in internalization of Gαs-GFP and uptake of cholera toxin B (Fig. 4, lower panel). Isoproterenol treatment resulted in a strong colocalization between Gαs-GFP and cholera toxin B within internalized vesicles. Numerous vesicles contained both cholera toxin B and internalized Gαs-GFP (arrows). This suggests that agonist activated Gαs-GFP is internalized from lipid raft domains of the plasma membrane, possibly by caveolae or raft mediated endocytosis.

Isoproterenol Stimulation Increases Gαs present in the Cytosol

Numerous studies examining the fate of Gαs upon receptor activation have indicated that Gαs is released into the cytosol (Ransnas et al., 1989; Thiyagarajan et al., 2002; Wedegaertner et al., 1996; Yu and Rasenick, 2002). The previous investigation utilizing Gαs-GFP demonstrated that isoproterenol treatment results in a dissociation of the fluorescent protein from plasma membrane and the activated construct increases localization in the cytosol similar to wild type Gαs (Yu and Rasenick, 2002). To further confirm this phenomenon, C6 cells were treated with β receptor agonist for 30 minutes and purified cytosol and membrane fractions were obtained and analyzed by immunoblotting for endogenous Gαs content. Gαs was found in the cytosol of both control and isoproterenol treated C6 cells (Fig. 5A). Isoproterenol treatment of C6 cells increased endogenous Gαs present in the cytosol by nearly 3 fold versus control. The integrated

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optical density (IOD) of Gαs immunoblots were quantitated by scanning densitometry and data pooled from four experiments (Fig, 5A, n=4; Con Pellet=1053±75; Con Cytosol=76±13; ISO Pellet=1002±68; ISO Cytosol=*193±22; *p<0.05 vs. Con Cytosol). Increased cytosolic localization of Gαs in C6 cells in response to receptor activation further confirms reports that Gαs undergoes a subcellular redistribution after activation.

Analysis of Gαs and B_2AR Localization in Lipid Rafts/Caveolae.

Colocalization of Gαs-GFP with the lipid raft marker cholera toxin B (Fig. 4) suggests that lipid rafts may play an important role in agonist induced internalization of Gαs. To assess biochemically the localization of endogenous Gαs in these membrane domains, C6 cells were treated with +/- isoproterenol and Triton X-100 detergent resistant raft/caveolae membranes and Triton X-100 soluble non-raft membranes were isolated by sucrose density gradient ultracentrifugation as described in Materials and Methods. The amount of endogenous Gαs in these membranes was determined by immunoblotting. Immunoblots were probed for both Gαs (Fig. 5B upper panel) and the protein caveolin-1 (Fig. 5B lower lanel), which is a positive marker found exclusively in lipid rafts/caveolae. Gαs was found in both detergent resistant raft/caveloae fractions and also Triton X-100 soluble membrane fractions. Thirty minutes of isoproterenol treatment resulted in a significant increase in Gαs protein present in the lipid raft/caveolae fractions, and a concomitant decrease in the Triton X-100 soluble membrane (non-raft) fractions (Fig. 5B). A time course of isoproterenol treatment was also performed and percent change in Gαs protein in the lipid raft/caveolae fractions was determined (Fig. 5C). Isoproterenol treatment resulted in increased Gαs localization in rafts by approximately 30% above control levels within 10 minutes (Fig 5C), This agonist induced increase of Gαs in

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rafts/caveolae indicates that Gαs moves into these membrane microdomains subsequent to agonist activation.

Similarly, the raft and non-raft membrane localization of the β_2AR was determined before and after agonist stimulation. The polyclonal β_2AR antibody recognized two bands between the 47 and 76 kDa markers, the lowest band was estimated to be approximately 62kDa. β_2AR immunoreactivity appeared enriched in the Triton X-100 soluble membranes, with a lesser portion detected in the caveolin enriched raft/caveloae fractions (Fig 5D). However, after 30 minutes of isoproterenol treatment, β_2ARs were significantly decreased in the raft/caveolae fractions by nearly 80 percent (Fig 5D). This decrease in β_2AR localization in rafts/caveolae is consistent with previous reports in other cell types demonstrating that activated β_2ARs leave these microdomains (Ostrom et al., 2001; Rybin et al., 2000). Collectively, these data demonstrate that agonist stimulation results in subtle shifting of Gαs and β_2AR in or out of raft/caveloae membranes during signaling,

Depletion of Membrane Cholesterol Prevents Gαs-GFP Internalization.

Increased localization of Gαs in lipid raft/caveolae fractions after isoproterenol exposure suggests these microdomains are important for Gαs trafficking. Depletion of cholesterol from cell membranes using the chelating agent methyl-β-cyclodextrin (CD) is commonly used to inhibit raft/caveolae mediated endocytosis (Nichols, 2003). Exposure of C6 cells to 10mM CD for 30 min. resulted in a profound decrease in the amount of endogenous Gαs located in lipid rafts/caveolae, and increased the amount of Gαs present in the soluble non-raft membranes (Fig 6A, top panel). Depletion of cholesterol with CD decreased both long and short forms of Gαs present in raft/caveolae fractions, while the amount of caveolin-1 was not affected (Fig 6A,

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lower panel). These results show that removing cholesterol from cell membranes results in a significant depletion of Gαs present in lipid rafts/caveolae. To ensure that CD was not toxic, cholesterol complexes were added back to CD treated cells as described in Materials and Methods. Adding cholesterol back to cells partially restored Gαs localization to the raft/caveloae fractions (Fig. 6B).

To investigate if CD can block Gαs-GFP internalization, C6 cells expressing Gαs-GFP were exposed to 10mM CD for 30 min., followed by isoproterenol stimulation during live cell imaging. A representative C6 cell shown in Fig. 6C demonstrates that agonist induced internalization of Gαs-GFP is prevented by cyclodextrin treatments. Gαs-GFP remained localized on the plasma membrane during agonist stimulation, and Gαs-GFP was not found in vesicles or punctate structures. Although CD treatment prevented Gαs-GFP internalization, it did not inhibit clathrin mediated endocytosis of transferrin (Fig 6C, top panel 3). In similar experiments, C6 cells expressing Gαs-GFP were initially treated with CD, and then cholesterol was delivered back to cells in the form of CD-cholesterol complexes. Images of a representative C6 cell demonstrate that CD effects are reversed when cells are provided cholesterol complexes to restore lipid rafts/caveloae (Fig 6C lower panel). Fluorescent images of C6 cells pooled from 10 independent experiments were quantitated for Gαs-GFP internalization after treatments. These data demonstrate that disrupting rafts with cyclodextrin prevents agonist induced internalization of Gαs-GFP, and this inhibition is reversible if cholesterol is delivered back to C6 cells. The blockade of Gαs-GFP internalization by inhibition of raft/caveolae endocytosis suggests that Gαs endocytosis is carried out through lipid rafts/caveolae.

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Quantification of Gαs-GFP Internalization in MCF-7 cells.

To quantitatively assess Gαs internalization in MCF-7 cells, Gαs-GFP expressing cells were treated with isoproterenol for 30 minutes, fluorescent images were obtained and the grey value intensity of Gαs-GFP present in the cytoplasm of cells was measured using NIH image as described in Materials and Methods. It is worth noting that this method of quantitating internalization detects the intracellular signal of Gαs-GFP, regardless of whether the fluorescence is cytoplasmic or vesicular in nature. Isoproterenol treatment significantly increased Gαs-GFP internalization versus control cells (Fig. 7). In contrast, cells co-transfected with Gαs-GFP and dominant negative K44E dynamin 1 did not show a significant internalization in response to agonist. Similarly, cells pre-treated with the lipid raft inhibitor methyl-β-cyclodextrin also did not show agonist mediated internalization of Gαs-GFP. Quantitative data are from images of MCF-7 cells pooled from 10 independent experiments. This quantitative assessment suggests that both Gαs-GFP internalization in vesicles and redistribution of Gαs-GFP into the cytoplasm require intact lipid rafts/caveolae and dynamin dependent endocytosis.

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DISCUSSION

To assess the real-time trafficking of Gαs during signal transduction we have utilized the well established approach of visualizing a GFP fusion protein, providing a convenient method for examining G protein trafficking in real-time (Hynes et al., 2004a; Janetopoulos and Devreotes, 2002; Yu and Rasenick, 2002). We have purposefully chosen to study Gαs trafficking by expressing Gαs-GFP at low levels in C6 and MCF-7 cells that express only endogenous β_2ARs. This model enables Gαs-GFP to become activated by only endogenous βARs, and we consider this approach preferable to overexpressing receptors. Real-time imaging of Gαs-GFP demonstrates that isoproterenol treatment results in a removal of Gαs-GFP from the plasma membrane and internalization of the protein within vesicles (Fig 1A and supplemental movie). Agonist induced internalization of Gαs-GFP appears to be dynamin 1 dependent, as over expression of the K44E dominant negative dynamin mutant prevented Gαs-GFP internalization (Fig. 2, Fig. 7). Data also demonstrate that Gαs-GFP redistributes into the cytoplasm after receptor stimulation (Fig. 3, Fig. 4), which is consistent with previous findings about both wild type Gαs and Gαs-GFP (Yu and Rasenick, 2002). Note that both endogenous Gαs and Gαs-GFP have an identical cellular distribution, and previously both indentically redistributed in response to isoproterenol (Yu and Rasenick, 2002). In addition, agonist significantly increased the content of endogenous Gαs in the cytosol of C6 cells (Fig. 5A), and this supports the many studies demonstrating a cytosolic redistribution of activated Gαs.

Unexpectedly, markers for early and recycling endosomes, as well as late endosomes/lysosomes did not colocalize with internalized vesicles containing Gαs-GFP (Fig. 3A, 3B, 3C). Since neither EEA-1 nor transferrin colocalized with Gαs-GFP, it is unlikely that early endosomes or recycling endosomes are involved in trafficking of Gαs during

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internalization. Consistent with these results, internalized vesicles containing Gαs-GFP did not colocalize with endocytosed β_2ARs (Figm 3D), which are known to traffic into early and recycling endosomes (Claing et al., 2002). Lack of colocalization of internalized Gαs-GFP with β_2ARs agrees with previous results showing that internalized Gαs does not colocalize with the receptors in endosomes (Hynes et al., 2004b; Wedegaertner et al., 1996). These results collectively suggest that internalized Gαs does not traffic in common compartments of the endocytic pathway.

Both lipid rafts and caveolae are cholesterol and glycolipid rich microdomains of the plasma membrane involved in a mode of endocytosis distinct from classical clathrin mediated endocytosis. Since internalized Gαs-GFP did not colocalize with markers for common endocytic compartments, we investigated the potential involvement of lipid rafts. Using the fluorescent marker cholera toxin B, which binds to and is internalized from rafts, microscopy demonstrates that internalized Gαs-GFP strongly colocalizes with cholera toxin B in vesicles in living cells (Fig. 4), suggesting that Gαs is internalized from raft microdomains. It is worth noting cholera toxin B may also label compartments such as early endosomes in some cell types (Torgersen et al., 2001), but cholera toxin B is endocytosed predominantly by non-clathrin mediated endocytosis (Nichols et al., 2001; Orlandi and Fishman, 1998; van Deurs et al., 2003). Colocalization of cholera toxin B with Gαs-GFP in internalized vesicles strongly suggests lipid rafts are involved in Gαs internalization.

To further support this observation, biochemical studies revealed that isoproterenol treatment of C6 cells significantly increased the level of endogenous Gαs located in Triton X-100 detergent resistant raft/caveloae fractions (Fig. 5B, 5C). The finding that in unstimulated cells, Gαs is present in rafts/caveolae is consistent with previous studies (Oh and Schnitzer,

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2001; Toki et al., 1999). Increased localization of Gαs in raft/caveolae fractions suggests Gαs moves into these membrane domains during receptor stimulation where it may subsequently become internalized. In contrast to this, it appears that activated β_2ARs leave the lipid raft/caveloae microdomains (Fig. 5D), data that are consistent with reports shown in the cardiomyocyte (Ostrom et al., 2001; Rybin et al., 2000). There appears to be cellular heterogeneity concerning β_2AR compartmentalization. β_2ARs appear to be evenly distributed between raft and non-raft fractions in cardiomyocytes, however in other tissues such as vascular smooth muscle or airway epithelia, β_2ARs are largely excluded from rafts (Ostrom and Insel, 2004). Our data show that in C6 glioma cells, β_2ARs are found predominantly in non-raft fractions (Fig. 5D). Although fractions examined do not account for all Gαs and β_2AR, Gαs appears enriched in the raft/caveolae domains, while β_2ARs are weighted to non-rafts. Considering that the ratio of β_2AR to Gαs is 1:100 in C6 cell membranes which we have calculated (Manier et al., 1992; Toki et al., 1999), this would increase the ratio of receptor to Gαs in the non-raft regions. It is unclear how these ratio differences of Gαs to β_2AR in the membrane domains contribute to βAR signaling. The increased association of Gαs with rafts, but removal of β_2ARs from rafts during signaling further supports the hypothesis that Gαs internalizes and traffics distinctly from the β_2AR.

Additional evidence supporting the hypothesis that rafts mediate Gαs internalization can be found from the studies using methyl-β-cyclodextrin. Incubation of C6 cells with cyclodextrin resulted in a profound decrease in Gαs located in rafts/caveolae, demonstrating the importance of cholesterol in targeting Gαs to these microdomains (Fig. 6A, 6B). In C6 cells pre-incubated with cyclodextrin prior to isoproterenol treatment, Gαs-GFP internalization was blocked without affecting transferrin internalization (Fig 6C). Noteably, the inhibitory effects of cyclodextrin

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were reversed by adding cholesterol back to cells, indicating cyclodextrin treatments are not toxic to the cells. It is noteworthy that in certain conditions, cyclodextrin has also been reported to inhibit clathrin mediated endocytosis in some cell lines (Subtil et al., 1999). However, as cyclodextrin did not prevent endocytosis of transferrin, it is unlikely that clathrin mediated endocytosis was inhibited by cyclodextrin in these experiments. Similar to C6 cells, cyclodextrin also prevented Gαs-GFP internalization in MCF-7 cells treated with isoproterenol (Fig. 7). These results support the conclusion that βAR stimulation promotes Gαs movement into lipid rafts, and that these microdomains are necessary for Gαs internalization.

A summary of experimental results and a working model for Gαs internalization is illustrated and described in Figure 8. This model proposes that Gαs becomes internalized within vesicles derived from lipid rafts and that Gαs trafficking is distinct from the β_2AR.

The agonist mediated internalization and trafficking of βARs is a well described phenomenon, however relatively little is understood about the mechanisms regulating heterotrimeric G protein internalization. We have focused on the trafficking of Gαs in this study, but very recent work demonstrates that Gβγ is also internalized in response to β agonist in HEK293 cells (Hynes et al., 2004b). Internalization of activated G proteins adds a substantial complexity to the regulated signaling of β adrenergic receptors, one that requires proper trafficking of both receptors and their cognate G proteins to maintain signaling fidelity. Recent experiments have shown PKA phosphorylated β_1ARs will preferentially internalize through caveolae/rafts (Rapacciuolo et al., 2003). While cells studied in this investigation express the β_2AR subtype, in future experiments it will be instructive to investigate if β_1ARs traffic together with internalized Gαs from rafts/caveloae.

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Lipid rafts/caveolae are typically thought of as membrane microdomains which spatially organize molecules to facilitate GPCR mediated signaling. However, this assumption likely depends on which G protein and receptor pathway are involved. Recently, Roth and co-workers demonstrated in C6 cells that 5HT2A/Gq coupled receptor pathways are dependent on caveolae and interactions with caveolin-1, suggesting caveolae promote 5HT2A/Gq signaling (Bhatnagar et al., 2004). In contrast to this, results in this report suggest Gαs in lipid rafts/caveolae may be removed from membrane signaling cascades. Treatment of C6 cells with antidepressant drugs results in a removal of Gαs from rafts/caveolae and an increase in cAMP synthesis, supporting the concept that shifting Gαs out of raft domains enhances cAMP signaling (Donati et al., 2001; Toki et al., 1999). Two previous studies in which lipid rafts/caveolae were disrupted by cyclodextrin revealed that depletion of rafts significantly increased isoproterenol stimulated cAMP production (Miura et al., 2001; Rybin et al., 2000). Increased cAMP production in cells depleted of rafts/caveolae is consistent with the notion that these domains are effective in silencing cAMP production. Thus, isoproterenol induced movement of Gαs into lipid rafts and its subsequent internalization may be involved in modulating cAMP production, however this has yet to be confirmed.

Internalization could also enable activated Gαs to interact with effectors at multiple intracellular sites. Several studies have indicated Gαs regulates endocytic trafficking. Gαs appears to be involved in the regulation of apical transport in liver epithelia (Pimplikar and Simons, 1993), and antibodies against Gαs prevent fusion of endosomal vesicles (Colombo et al., 1994). Recently, Gαs has been implicated in regulating the trafficking and degradation of EGF receptors through interactions on endosomal vesicles (Zheng et al., 2004). Isoproterenol induced internalization of Gαs from rafts is consistent with these findings, and may be an event enabling

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activated Gαs to traffic into the cellular interior to regulate endocytic pathways. Lastly, Gαs has also been shown to associate with cytoskeletal elements and Gαs is capable of activating the GTPase of tubulin and increasing microtubule dynamics (Roychowdhury et al., 1999; Sarma et al., 2003). Thus, internalization of Gαs and association with the microtubule cytoskeleton could be a mechanism facilitating agonist mediated cell shape changes. In future studies, it will be informative to investigate the roles of both the actin and microtubule cytoskeletons in vesicular trafficking of Gαs.

In summary, this report reveals that activated Gαs-GFP translocates away from the plasma membrane by endocytosis, and that Gαs trafficking is separate from β_2ARs. Activated Gαs increases its localization in lipid rafts/caveolae during agonist treatment and internalization occurs from lipid raft microdomains of the plasma membrane that is dynamin 1 dependent. It is suggested that agonist induced internalization of Gαs and association with vesicles may alter cAMP production, while enabling Gαs to participate in intracellular signaling events.

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FOOTNOTES

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FIGURE LEGENDS

FIG 1. β-adrenergic receptor stimulation promotes Gαs-GFP internalization in living C6 and MCF-7 cells. C6 and MCF-7 cell lines were transiently transfected with Gαs-GFP. 24 hours after transfection, living cells were stimulated with 10μM isoproterenol, and real-time trafficking of Gαs-GFP in response to agonist was assessed using digital fluorescence microscopy as described in Materials and Methods. Living cells were imaged in real time and individual frames are shown prior to and after agonist treatment at the indicated times. ***A,*** representative C6 cell was treated for 10 minutes with isoproterenol, arrows indicate regions where Gαs-GFP dissociates from the plasma membrane. ***B,*** representative MCF-7 cell treated for 10 minutes with isoproterenol. During agonist treatment, Gαs-GFP present in membrane extensions was rapidly reorganized and vesicles containing the protein appeared to form from these structures. Over the 8 minute period of stimulation, many vesicles containing the protein were found within the cytoplasm, and these vesicles trafficked retrogradely into the cell interior. Inset images are shown 2 minutes and 8 minutes after addition of isoproterenol, and arrows indicate retrograde trafficking of a single Gαs-GFP containing vesicle. See **Fig. 1B supplemental video** online for the real-time video of these images. Scale bars = 10μm.

FIG 2. K44E dominant negative dynamin overexpression prevents agonist induced internalization of Gαs-GFP. C6 cells were transiently co-transfected with Gαs-GFP and dominant negative K44E dynamin 1 constructs. 16 hours after transfection, living cells were stimulated with 10μM isoproterenol and Gαs-GFP trafficking was imaged in real time using digital fluorescence microscopy. Individual frames of Gαs-GFP from a representative cell are shown prior to and after agonist exposure at the indicated times. Scale bar = 10μm.

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FIG 3. Internalized Gαs does not traffic into common endocytic compartments or colocalize with β_2 adrenergic receptors. ***A and B,*** MCF-7 cells were transiently transfected with Gαs-GFP, and cells were stimulated for 30 minutes with 10μM isoproterenol to promote internalization. Cells were then fixed and immunocytochemistry was performed using antibodies against the early endosome antigen 1 protein (EEA1) or the lysosome associated membrane protein (LAMP-1). ***A,*** representative MCF-7 cell showing no colocalization between internalized Gαs-GFP (green) and early endosomes labeled with EEA-1 antibodies (red). ***B,*** representative MCF-7 cell reveals no colocalization between Gαs-GFP (green) with late endosomes and lysosomes labeled with LAMP-1 antibodies (red). ***C,*** Gαs-GFP expressing lcells were pre-incubated with the recycling endosome marker transferrin Texas red ligand at 4º C. Cells were warmed to 37º C in the presence of 10μM isoproterenol, and colocalization between Gαs-GFP (green) with transferrin (red) was assessed in living cells. Two representative MCF-7 cells after 15 minutes of isoproterenol treatment reveal no colocalization of Gαs-GFP with transferrin. ***D,*** MCF-7 cells expressing Gαs-GFP were stimulated for 30 minutes with 10μM isoproterenol to promote internalization. Cells were fixed and processed for immunocytochemistry using a β_2AR antibody. Two representative cells are shown, merged image indicates no colocalization between Gαs-GFP (green) and the β_2AR (red). Scale bars for all images = 10μm.

FIG 4. Internalized Gαs-GFP co-localizes with the lipid raft marker cholera toxin B in living MCF-7 cells. MCF-7 cells were transiently transfected with Gαs-GFP and living cells were pre-incubated with the lipid raft marker, fluorescent cholera toxin B at 4º C as described in

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Materials and Methods. Cells were then warmed to 37º C in the presence of 10μM isoproterenol, and colocalization of Gαs-GFP (green) with cholera toxin B subunit (red) was assessed using digital fluorescence microscopy. ***Top panel,*** representative cell expressing Gαs-GFP (green) and labeled with cholera toxin B (red) at 4º C before isoproterenol stimulation. Gαs-GFP and cholera toxin B strongly co-localize at the plasma membrane. ***Lower panel,*** Merged image of two representative cells reveals strong colocalization of Gαs-GFP with cholera toxin B in internalized vesicles (arrows) after isoproterenol stimulation. Scale bars = 10μm.

FIG 5. Isoproterenol treatment increases endogenous Gαs localization in both cytosol and lipid rafts, while activated β_2-adrenergic receptors leave lipid rafts. ***A,*** C6 glioma cells were treated with +/- 10μM isoproterenol for 30 minutes and membrane and cytosolic fractions were obtained as described in Materials and Methods. The quantity of endogenous Gαs in the membrane pellet (P) and in cytosolic soluble fractions (S) was determined by western blotting. A representative blot (n=4) of Gαs protein indicates isoproterenol increases the amount of Gαs present in the cytosol, without changing the total amount of Gαs. ***B,*** Agonist stimulation increases Gαs localization in rafts. C6 glioma cells were treated with +/- isoproterenol for 30 minutes. Detergent insoluble lipid rafts/caveolae (Raft) as well as non-raft detergent soluble membranes (TXSol) were obtained by sucrose density gradient fractionation (see "Materials and Methods"). A representative blot of Gαs protein is shown (upper panel), the same blot was re-probed for the lipid raft/caveolae marker protein caveolin-1 (lower panel). The figure is a quantitation of Gαs protein obtained by scanning densitometry and is presented as percent control (n=3). ***C,*** Time course of isoproterenol treatment and Gαs localization in the lipid raft/caveolae membrane fractions. C6 glioma cells were stimulated with isoproterenol for 10, 30

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and 60 minutes and rafts obtained as in B. A representative blot of Gαs protein in lipid rafts after isoproterenol treatment is shown (upper panel), as well as caveolin-1 protein (lower panel). The figure shows percent change in Gαs protein above control in the lipid raft/caveolae membrane fractions from four independent experiments. ***D,*** β_2-adrenergic receptors leave lipid rafts after agonist stimulation. C6 cells were treated, membrane fractions obtained as in B, and immunoblotting performed for the β_2AR. A representative blot of β_2AR protein in these fractions before and after isoproterenol treatment is shown (upper panel), and the same blot re-probed for caveolin-1 protein (lower panel). The figure is a quantitation of β_2AR protein obtained by scanning densitometry and is presented as percent control (n=3). Data are represented as the mean ± SEM. *, $p<0.05$ versus control. IOD = integrated optical density. Approximate molecular weight markers are shown in kDa.

FIG 6. Disruption of lipid rafts prevents Gαs-GFP internalization. ***A,*** C6 cells were incubated with +/- 10mM methyl-β-cyclodextrin (CD) for 30 minutes, or with cyclodextrin followed by CD-cholesterol complexes (CD+CHOL) for 90 minutes to restore cholesterol back to the cells. 1% Triton X-100 soluble membranes (TXSol) and insoluble rafts/caveolae (Raft) were obtained as described in Figure 5. A representative immunoblot shows endogenous Gαs protein in these fractions (upper panel), and the lipid raft/caveolae marker caveolin-1 (lower panel). Approximate molecular weight markers are shown. ***B,*** Quantitation of Gαs protein by scanning densitometry and presented as percent control (n=3). ***C, upper panel,*** C6 cells expressing Gαs-GFP were treated with (CD) for 30 min., washed, and pre-incubated with transferrin Texas red ligand at 4º C. Cells were warmed to 37º C in the presence of 10μM isoproterenol (ISO), and Gαs-GFP and transferrin trafficking were assessed in living cells using

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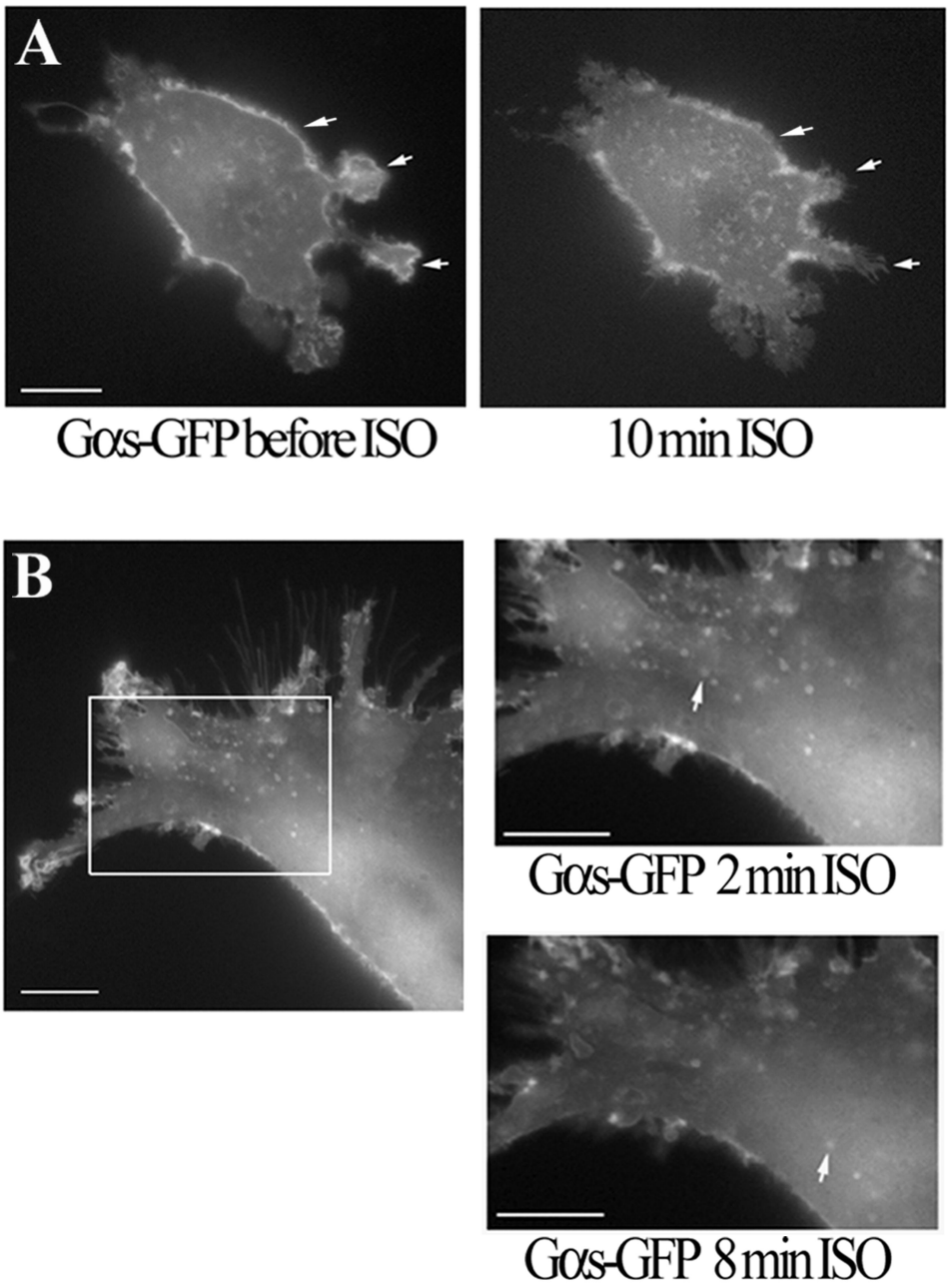
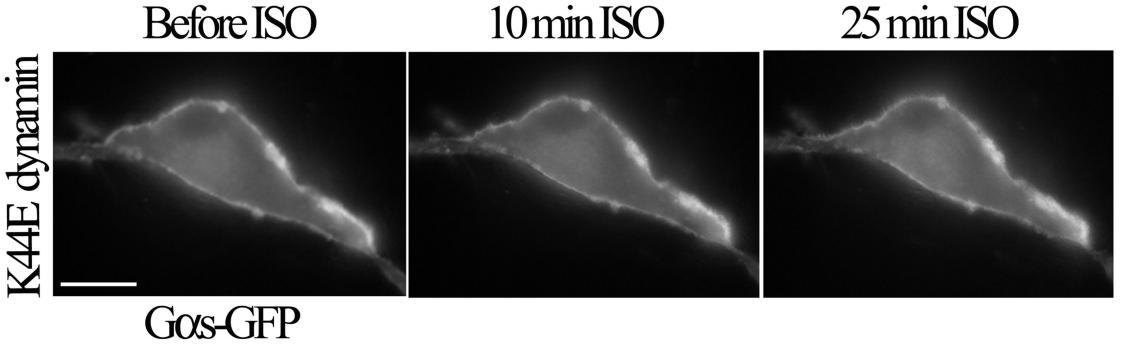
fluorescence microcopy. Images of a representative C6 cell reveals that CD treatment prevents agonist induced internalization of Gαs-GFP, but not internalization of transferrin. ***C, lower panel,*** C6 cells expressing Gαs-GFP were initially treated with CD then with CD-cholesterol complexes to restore lipid rafts. Images of a representative C6 cell reveal that CD effects can be reversed by adding cholesterol back to the cells as isoproterenol treatment of these cells restored Gαs-GFP internalization. ***D,*** Quantitation of Gαs-GFP internalization in C6 cells. The grey value intensity of Gαs-GFP present in the cytoplasm of cells was measured using NIH image as described in Materials and Methods. Data are from images of cells pooled from 10 independent experiments (n=10). Scale bars = 10μm. Data are represented as the mean ± SEM. *, $p<0.05$ versus control. IOD = integrated optical density.

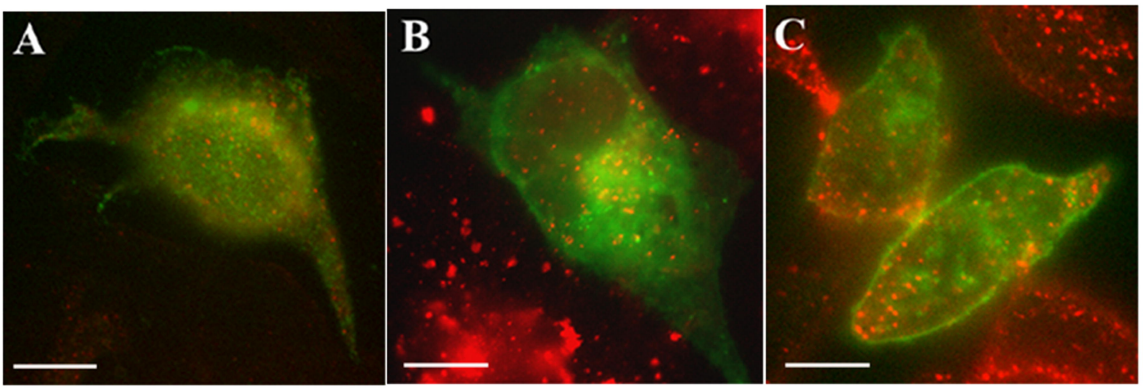
FIG 7. Quantitation of Gαs-GFP internalization. MCF-7 expressing Gαs-GFP were treated with +/- isoproterenol for 30 minutes and fluorescent images were obtained. The grey value intensity of Gαs-GFP present in the cytoplasm of cells was measured using NIH image as described in Materials and Methods. Isoproterenol (ISO) treatment significantly increases Gαs-GFP in the cytoplasm of cells, but this internalization is prevented when cells are co-transfected with dominant negative K44E Dynamin 1 (K44E Dyn), or in cells treated with the lipid raft inhibitor methyl-β-cyclodextrin (CD). Data are from images of MCF-7 cells pooled from 10 independent experiments (n=10). *, $p<0.05$ versus control.

FIG 8. A model for agonist induced internalization of Gαs. Upon isoproterenol binding to the β_2AR, Gαs exchanges GDP for GTP resulting in dissociation of the G proteins from the receptor, and Gαs becomes activated. Within seconds, the receptor becomes phosphorylated by G protein

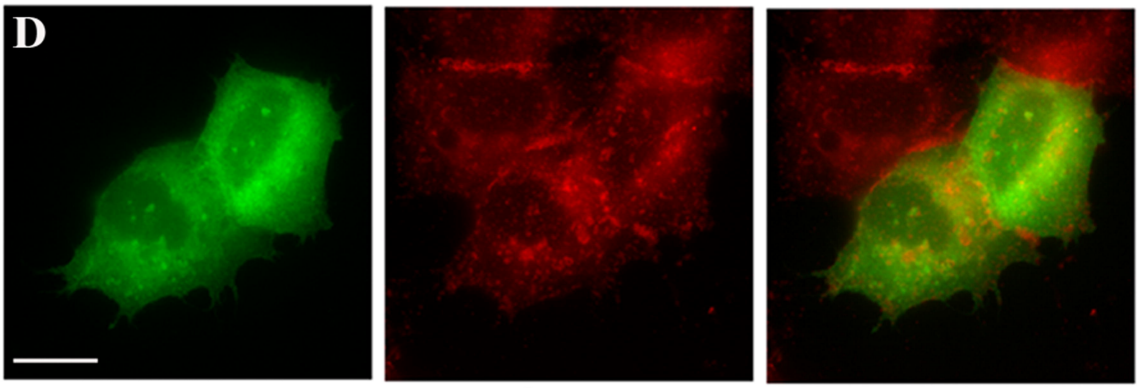
coupled receptor kinases (GRKs), and through interactions with β-arrestin, the receptor is recruited into clathrin coated pits and trafficked into early endosomes where ligand is removed by acidic pH (Claing et al., 2002). The receptor trafficks into classical endosomes containing EEA-1 and transferrin markers. After agonist activation, the β_2AR leaves lipid raft/caveolae microdomains (Fig. 5D). In contrast to this, activated Gαs increases its association with lipid raft microdomains (Fig. 5B, 5C), and it is internalized within distinct vesicles derived from those domains. These vesicles containing Gαs positively label with the lipid raft marker cholera toxin B (Fig. 4). Gαs internalization from lipid rafts is dynamin 1 dependent (Fig. 2, Fig. 7) and can be inhibited by the lipid raft disrupting drug cyclodextrin (Fig. 6, Fig. 7). After receptor activation, Gαs is also released into the cytosol (Fig. 5A), and this release may occur from either lipid rafts at the plasma membrane or from internalized vesicles. Taken together, this model suggests that activated Gαs becomes internalized within vesicles derived from lipid rafts and that Gαs trafficking is distinct from the β_2AR.


A
Gαs-GFP before ISO
10 min ISO
B
Gαs-GFP 2 min ISO
Gαs-GFP 8 min ISO

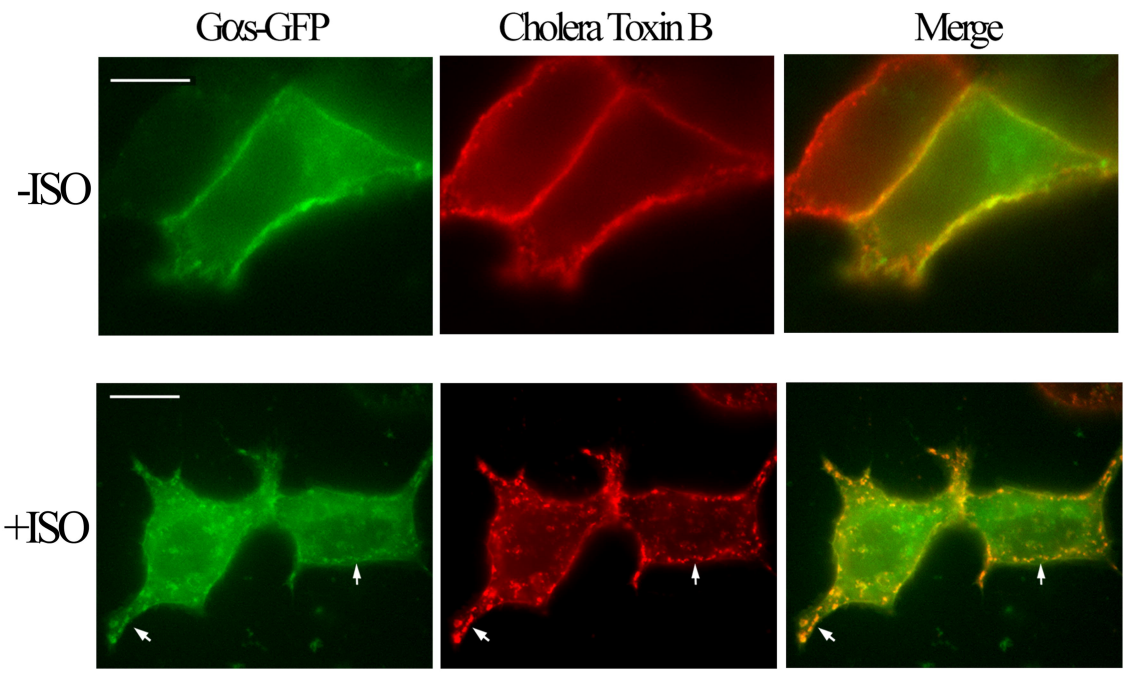

Before ISO
10 min ISO
25 min ISO
K44E dynamin
Gαs-GFP

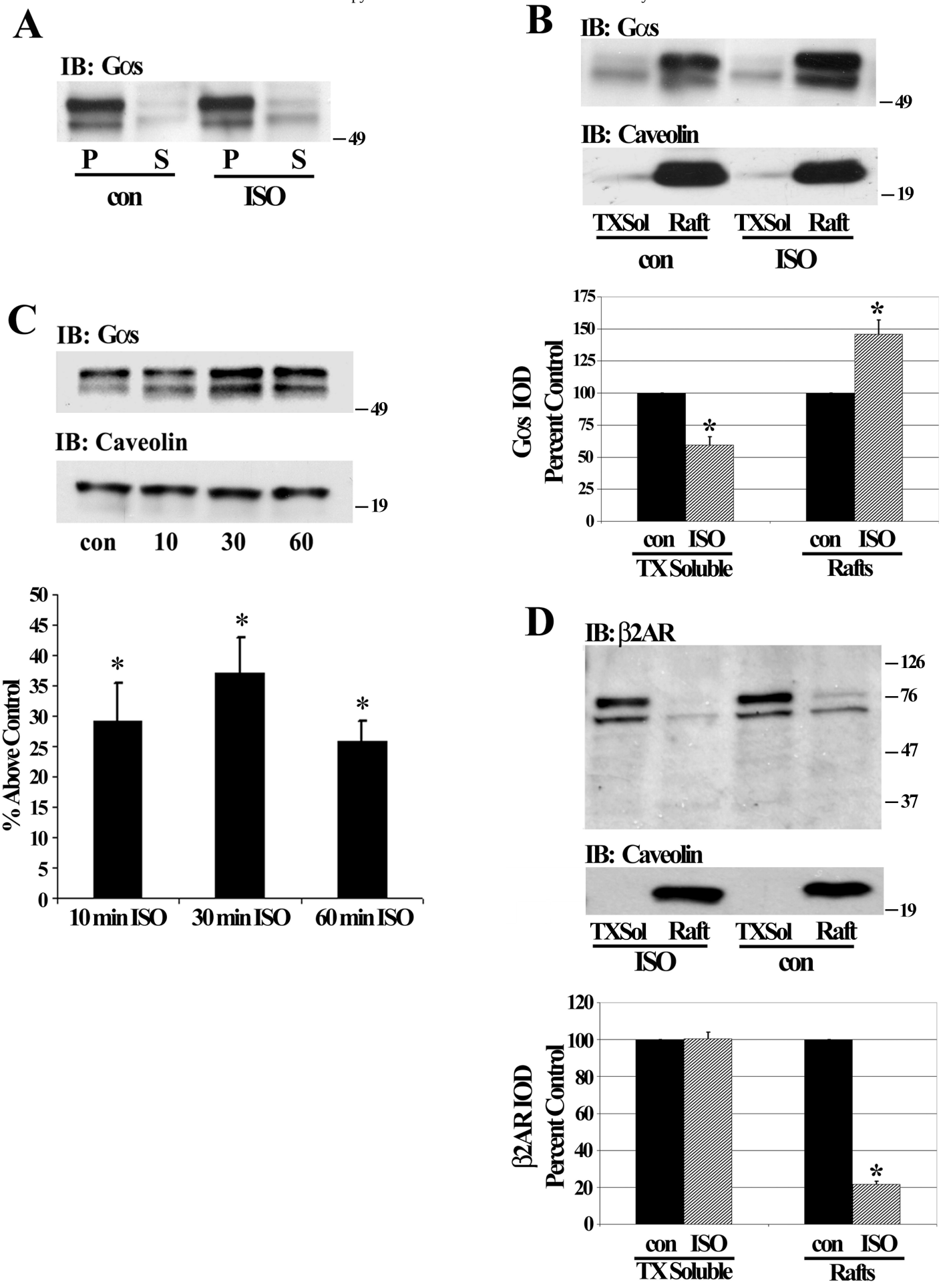


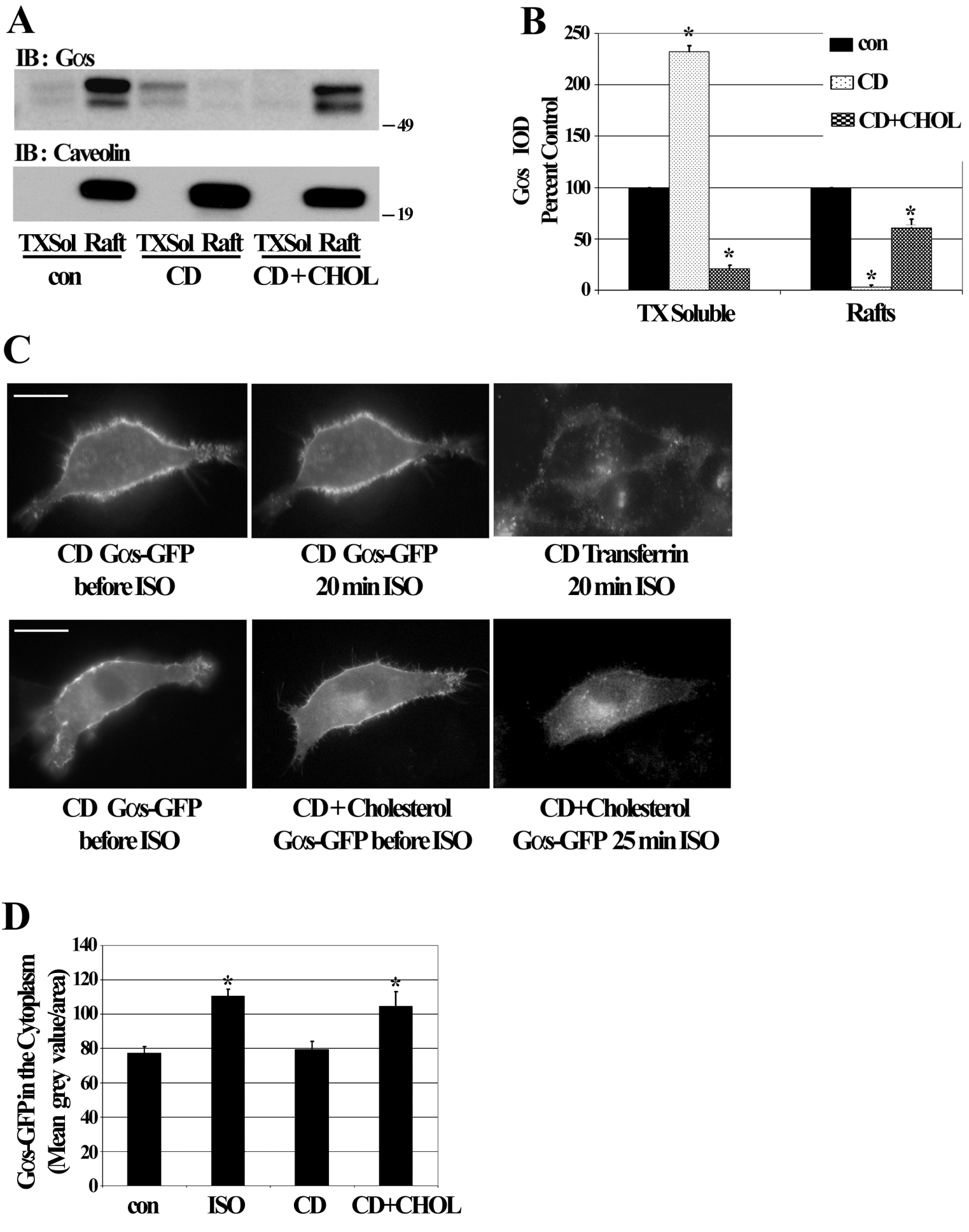
Gαs-GFP and EEA-1 Gαs-GFP and LAMP-1 Gαs-GFP and Transferrin

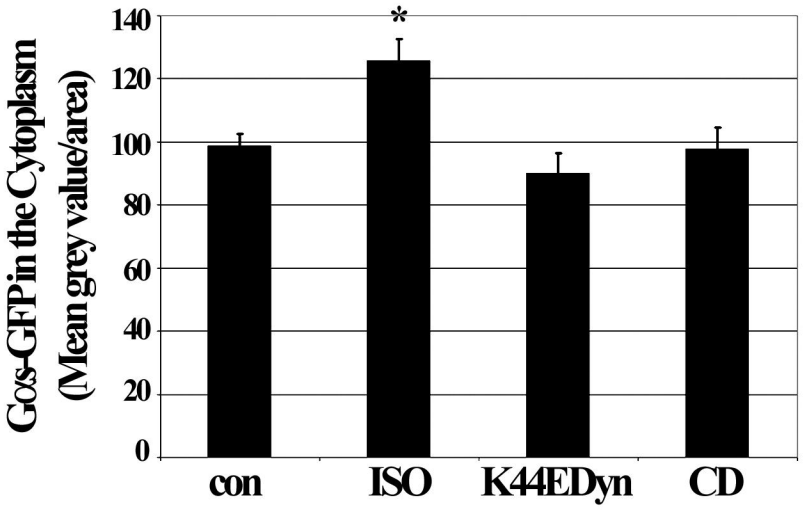


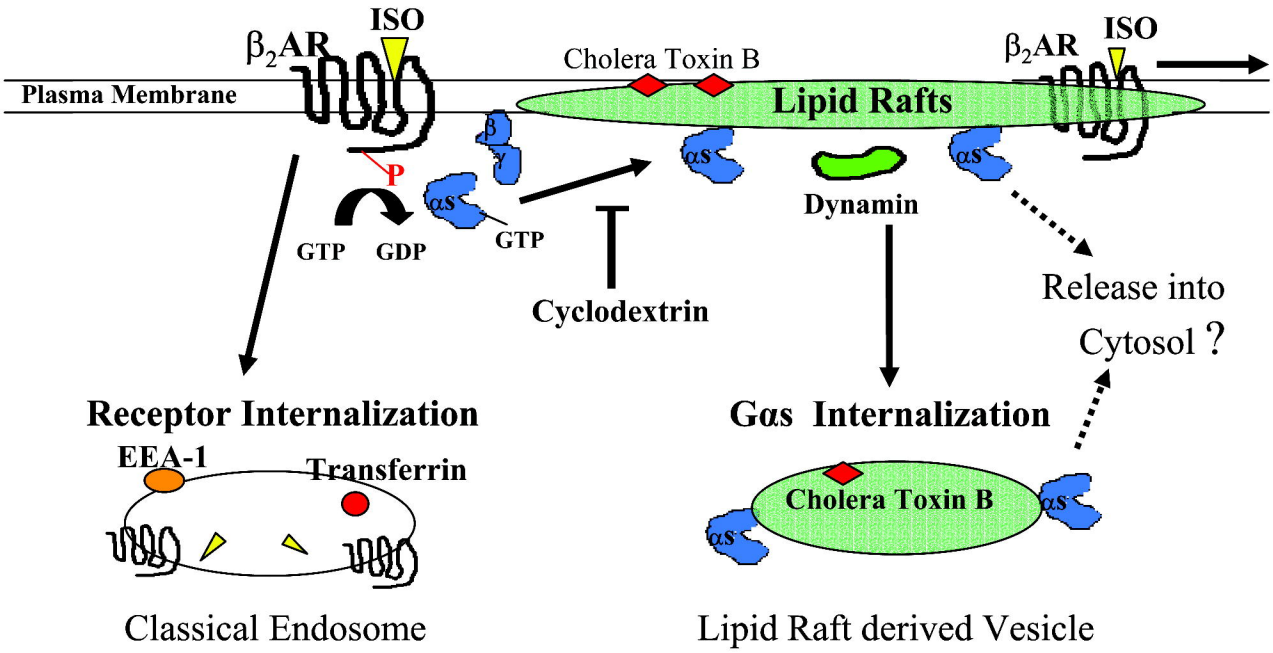
Gαs-GFP β_2-AR Merge


Gαs-GFP
Cholera Toxin B
Merge
-ISO
+ISO




A
IB: Gαs
49
IB: Caveolin
19
TXSol Raft
con
TXSol Raft
CD
TXSol Raft
CD+CHOL
B
Gαs IOD
Percent Control
250
200
150
100
50
0
con
CD
CD+CHOL
TX Soluble
Rafts
C
CD Gαs-GFP
before ISO
CD Gαs-GFP
20 min ISO
CD Transferrin
20 min ISO
CD Gαs-GFP
before ISO
CD+Cholesterol
Gαs-GFP before ISO
CD+Cholesterol
Gαs-GFP 25 min ISO
D
Gαs-GFP in the Cytoplasm
(Mean grey value/area)
140
120
100
80
60
40
20
0
con
ISO
CD
CD+CHOL


Gαs-GFP in the Cytoplasm
(Mean grey value/area)
140
120
100
80
60
40
20
0
*
con
ISO
K44EDyn
CD


ISO
β2AR
Plasma Membrane
Cholera Toxin B
Lipid Rafts
β
γ
αs
P
GTP
GDP
GTP
Cyclodextrin
Dynamin
αs
β2AR
ISO
Release into
Cytosol ?
Receptor Internalization
EEA-1
Transferrin
Gαs Internalization
Cholera Toxin B
αs
αs
Classical Endosome
Lipid Raft derived Vesicle