Live Cell Imaging of Gs and the β2-Adrenergic Receptor Demonstrates That Both αs and β1γ7 Internalize upon Stimulation and Exhibit Similar Trafficking Patterns That Differ from That of the β2-Adrenergic Receptor*‡

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To visualize and investigate the regulation of the localization patterns of Gs and an associated receptor during cell signaling, we produced functional fluorescent fusion proteins and imaged them in HEK-293 cells. αs-CFP, with cyan fluorescent protein (CFP) inserted into an internal loop of αs, localized to the plasma membrane and exhibited similar receptor-mediated activity to that of αs. Functional fluorescent β1γ7 dimers were produced by fusing an amino-terminal yellow fluorescent protein (YFP) fragment to β1(YFP-N-β1) and a carboxyl-terminal YFP fragment to γ7 (YFP-C-γ7). When expressed together, YFP-N-β1 and YFP-C-γ7 produced fluorescent signals in the plasma membrane that were not seen when the subunits were expressed separately. Isoproterenol stimulation of cells co-expressing αs-CFP, YFP-N-β1/YFP-C-γ7, and the β2-adrenergic receptor (β2AR) resulted in internalization of both fluorescent signals from the plasma membrane. Initially, αs-CFP and YFP-N-β1/YFP-C-γ7 stained the cytoplasm diffusely, and subsequently they co-localized on vesicles that exhibited minimal overlap with β2AR-labeled vesicles. Moreover, internalization of β2AR-GFP, but not αs-CFP or YFP-N-β1/YFP-C-γ7, was inhibited by a fluorescent dominant negative dynamin 1 mutant, Dyn1(K44A)-mRFP, indicating that the Gs subunits and β2AR utilize different internalization mechanisms. Subsequent trafficking of the Gs subunits and β2AR also differed in that vesicles labeled with the Gs subunits exhibited less overlap with Rab11-labeled endosomes and greater overlap with RhoB-labeled endosomes. Because Rab11 regulates traffic through recycling endosomes, co-localization of αs and β1γ7 on these endosomes may indicate a means of recycling specific αsβγ combinations to the plasma membrane.

Multiple G protein signaling pathways operate in individual cells to maintain homeostasis and to bring about responses to external stimuli such as growth and differentiation. An important, but unresolved issue is how the specificity of these pathways is maintained among so much complexity. 23 G protein α subunits, 5 β subunits, and 12 γ subunits have been identified in mammals (1), which could give rise to more than 1300 combinations. However, inactivation of specific G protein subunits in vivo using antisense (2–6) and riboynase (7, 8) strategies has demonstrated a remarkable specificity of interaction between receptors, αγγγ combinations, and effectors. For instance, riboynase-mediated suppression of γ7 in HEK-293 cells specifically reduces expression of β7 and disrupts activation of Gs by β-arrenergic and D1 dopamine receptors, but not by prostaglandin E1 and D2 dopamine receptors (8, 9). Moreover, knockout of γ7 in the mouse results in behavioral changes and reductions in the level of αs in the striatum (10).

One mechanism by which signaling specificity appears to be regulated is at the level of subcellular compartmentalization, which can facilitate or impair interactions between proteins expressed in the same cell (11, 12). However, in the case of protein complexes such as Gs, for which the localization patterns of the α and βγ subunits have been reported to change upon activation, it is not clear how specificity can be maintained. The Gs subunits associate with the plasma membrane as a result of fatty acid modifications and association with each other. Targeting of β subunits to the plasma membrane requires association with prenylated γ subunits (13) and is facilitated by association with α subunits (14). Similarly, αs attaches to the plasma membrane as a result of amino-terminal palmitoylation (15, 16) and association with β7 (17). Activation of Gs results in depalmitoylation of αs (18), and studies using immunohistochemistry (19) and an αs-GFP fusion protein (20) have demonstrated activation-dependent movement of αs from the plasma membrane to the cytoplasm. Activation-dependent changes in βγ localization have not been imaged in cells, but subcellular fractionation indicated that βγ redistributes from the plasma membrane to low density microsomes upon stimulation of β-arrenergic receptors (21). In the face of these localization changes, it is not clear how specific αsβγ combinations can be preserved throughout multiple signaling cycles.

To address this issue, we have performed real time imaging of a Gs heterotrimer, αsβ1γ7, which mediates signaling from the β2AR to adenylyl cyclase (7, 8), in isoproterenol-stimulated HEK-293 cells. αs was visualized using an internally tagged αs-CFP fusion protein that has comparable activity to that of αs, whereas β1 and γ7 were imaged exclusively in the form of β1γ7 complexes using the strategy of BiFC (22). BiFC involves the production of a fluorescent signal by two nonfluorescent fragments of YFP when they are brought together by interactions between proteins fused to each fragment. When expressed

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The abbreviations used are: GFP, green fluorescent protein; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; mRFP, monomeric red fluorescent protein; β2AR, β2-adrenergic receptor; BiFC, bimolecular fluorescence complementation; GTPyS, guanosine 5′-O-(3-thiotriphosphate); EGFP, enhanced green fluorescent protein.

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Together, fusion proteins consisting of an amino-terminal YFP fragment fused to \( \beta_1 \) and a carboxyl-terminal YFP fragment fused to \( \gamma \) produce a fluorescent signal that is not obtained with either subunit alone (23). Our results indicate that both \( \alpha_s \) and \( \beta_1 \gamma \) internalize upon activation, showing significant localization on intracellular vesicles that are distinct from \( \beta_2 \gamma \)-labeled vesicles. Moreover, internalization of the \( \beta_2 \gamma \), but not \( \beta_1 \gamma \), is inhibited by the antimitotic drug nocodazole, indicating that the G protein subunits dissociate from the receptor upon activation, and utilize a different mechanism for internalization from that of the receptor. Compared with vesicles labeled with the \( \beta_2 \gamma \), vesicles labeled with \( \alpha_s \) and \( \beta_1 \gamma \) exhibited less overlap with Rhod-labeled endosomes and greater overlap with Rab11-labeled endosomes. Overlap of \( \alpha_s \) and \( \beta_1 \gamma \) with each other on these Rab11-labeled endosomes may indicate a means of recycling specific \( \alpha \beta \gamma \) combinations to the plasma membrane, because Rab11 regulates trafficking through the pericentriolar recycling endosome (24). This approach of simultaneously imaging the \( \alpha \) and \( \beta \gamma \) components of heterotrimeric G proteins in live cells will have many applications in elucidating the roles of specific \( \alpha \beta \gamma \) combinations in particular signaling pathways.

EXPERIMENTAL PROCEDURES

Production of Fluorescent Fusion Proteins—\( \alpha_s \)-CFP in the expression vector pCDNAI/Amp (Invitrogen) was generated from the long splice cDNA (25) containing the EE epitope (26) to compare polyacrylamide electrophoresis (10%), transferred to nitrocellulose, and probed with a monoclonal antibody to the EE epitope (26) to compare \( \alpha_s \)-CFP and \( \alpha_s \)-GFP. The antigen-antibody complexes were detected according to the ECL Western blotting protocol (Amersham Biosciences). Chemiluminescence was imaged using a Lumi-Imager (Roche Applied Science).

Imaging of Fluorescent Fusion Proteins—HEK-293 cells were plated at a density of 10^4 cells per well on Lab-Tek II, 4-well chamber coverslips and transiently transfected using 0.25 \( \mu \)g of LipofectAMINE 2000 reagent. Plasmids were transfected using the following amounts: \( \alpha_s \) and \( \alpha_s \)-CFP, 0.15 \( \mu \)g; \( \beta_2 \gamma \), \( \beta_2 \gamma \)-GFP, and \( \gamma \), and \( \gamma \)-CFP, 0.075 \( \mu \)g; \( \beta_2 \gamma \), \( \beta_2 \gamma \)-AR-GFP, and \( \beta_2 \gamma \)-AR-CFP, 0.05 \( \mu \)g; mRFP, CFP-Mem, \( \gamma \)-CFP, RhB, YFP-RhB, CFP-Rab11, and YFP-Rab11, 0.0125 \( \mu \)g; Dynamin-1-K44A-mRFP, 0.3 \( \mu \)g. Cells were imaged 2 days after transfection using a Zeiss LSM 61 confocal 200 fluorescence microscope under the control of IPLab software (Scanalytics) as described (23). Using the motorized x-y-z stage, time course images of cells located at 5–6 positions in the well were collected simultaneously. Cells selected for imaging expressed all of the labeled proteins, had a clearly delineated plasma membrane, and had a region of cytoplasm that was generally free of vesicles or other structures. Individual exposure times were optimized for each cell and color channel. Images for each color channel and DIC were collected at each position in the well every 60 s. Following the second time point, a stimulus of 200 \( \mu \)M isoproterenol was added to the well, resulting in a final concentration of 10 \( \mu \)M, and images were collected for 30 min. For each experimental condition, cells were imaged from plates transfected on 3 different days.

Image Analysis—Time course images were analyzed using IPLab software (Scanalytics Inc.). Before analysis, instrument background was subtracted from the images and corrections were made for bleaching so that the average intensity of the image was constant throughout the time course (see Supplemental Material). The in-focus membrane and vesicle features visible in the images were superimposed on a varying background of diffuse intensity because of out of focus, as well as soluble labeled protein. The images were processed to remove the low resolution diffuse background to produce a high resolution image that was used to identify groups of adjacent pixels or “segments” that corresponded to cellular features such as the plasma membrane and intracellular vesicles (see Supplemental Material). “Segments” defined as areas between signals in the plasma membrane and in the cytoplasm, a “border” centered on the plasma membrane, 6–10 pixels wide (0.6–1.0 \( \mu \)m), was drawn around the edge of the cell using a Cintiq pen based display screen (Wacom).

Changes in the plasma membrane intensity of labeled proteins were measured in cells co-expressing a membrane marker (YFP-Mem or CFP-Mem) that was used to segment membrane pixels and correct for intensity changes due to changes in cell shape. A segment of pixels covering a length of the plasma membrane was identified from the high resolution image of the membrane marker, using a portion of the border as a mask (Fig. 5). The average intensities of these pixels in the background and bleached-out areas were normalized using the in-focus membrane and membrane marker were determined. The membrane marker intensity values were normalized to a starting value of one and the labeled protein intensity values were divided by the normalized membrane marker values. This corrected for changes in cell shape during the time course, because the distribution of the membrane marker did not change in response to agonist stimulation. The corrected labeled protein intensities were normalized to a starting value of one and averaged with values from multiple cells. Images of unstimulated cells analyzed in this manner showed no response or drift in signal (Fig. 3A). Cells were designated as non-responders if the plasma membrane intensity of the labeled protein dropped less than 3% during the 30 min time course. Only images from the inside edge of the cell border were segmented and the areas of these segments were calculated using the high resolution images. The area of overlap between vesicles labeled with two different proteins was defined as the area of pixels that was segmented for both proteins. Because the images were acquired sequentially, vesicle movement reduced the measured overlap for vesicles that co-localized. Changes in cell shape in addition to drifting along the cell border, the nucleus was traced using the DIC image. Vesicle pixels for the labeled proteins were segmented as described above. The cytoplasm segment was defined as pixels within the inside edge of the cell border, outside of the nucleus, and not part of a vesicle segment.
FIG. 1. Production of a functional $\alpha_\gamma$-CFP fusion. A, model of $\alpha_\gamma$-CFP. The structure of CFP, based on that of GFP (79), is green. The helical domain of $\alpha_\gamma$-GTP$S$ (80) is pink and the GTPase domain is light blue. GTP$S$ is yellow. The SGGGGS linkers between CFP and $\alpha_\gamma$ are shown schematically in dark blue.

B, $\alpha_\gamma$ and $\alpha_\gamma$-CFP are expressed at similar levels in HEK-293 cell membranes. Cells were transfected with plasmid encoding $\alpha_\gamma$ (lane 1), $\alpha_\gamma$-CFP (lane 2), or vector (pcDNA1/Amp) (lane 3). Similar results were obtained in two additional experiments.

C, $\alpha_\gamma$-CFP and $\alpha_\gamma$ stimulate cAMP formation to similar extents in response to receptor activation. HEK-293 cells were transfected with 0.2 g of plasmid encoding the porcine $\alpha_\gamma$-adrenergic receptor (81). In addition, the cells were transfected with a total of 0.5 g of plasmid consisting of either vector alone (pcDNA1/Amp) or the indicated amounts of plasmid encoding $\alpha_\gamma$ (circles) or $\alpha_\gamma$-CFP (squares) plus varying amounts of vector to keep the total amount of transfected plasmid constant. cAMP was measured in the presence (filled symbols) or absence (open symbols) of 10 nM UK-14,304. Values represent the means of triplicate determinations ± S.D. from a single experiment. Similar results were obtained in two additional experiments.

D, $\alpha_\gamma$-CFP stimulates cAMP formation in response to receptor activation to a greater extent than a previously described $\alpha_\gamma$-GFP construct (20). HEK-293 cells were transfected as described for C using plasmid encoding $\alpha_\gamma$-CFP (circles) or $\alpha_\gamma$-GFP (squares). cAMP was measured in the presence (filled symbols) or absence (open symbols) of 10 nM UK-14,304. Values represent the means of triplicate determinations ± S.D. from a single experiment. Similar results were obtained in two additional experiments.

FIG. 2. $\alpha_\gamma$-CFP and YFP-N-$\beta$/YFP-C-$\gamma_\gamma$ internalize in response to stimulation with 10 nM isoproterenol and exhibit substantial co-localization on vesicles. HEK-293 cells were transfected with plasmids expressing $\alpha_\gamma$-CFP, YFP-N-$\beta$/YFP-C-$\gamma_\gamma$, $\beta_2$AR, and mRFP (cytoplasm marker, not shown). Prior to stimulation, $\alpha_\gamma$-CFP and YFP-N-$\beta$/YFP-C-$\gamma_\gamma$ localized to the plasma membrane (top row), and following stimulation, initially appeared in the cytoplasm (second row, 3 min) and then localized to vesicles (third row, 24 min). In this and subsequent figures, colors used in the merge images are listed following the construct name. Analysis of vesicle segments revealed a high degree of overlap (in yellow) of $\alpha_\gamma$-CFP (red) and YFP-N-$\beta$/YFP-C-$\gamma_\gamma$ (green) vesicles. The cytoplasm segment is shown in gray, and the cell border in blue. The video for this figure is available under Supplemental Materials. YN indicates YFP-N and YC indicates YFP-C. Bar = 10 μm.
The average intensities of the cytoplasm segments in the background- and bleach-corrected images of the labeled protein and the cytoplasm marker were determined. The cytoplasm marker intensity values were normalized to a starting value of one. The labeled protein intensity values were divided by the normalized cytoplasm marker values to correct for changes in cell shape during the time course, because the distribution of the cytoplasm marker did not change in response to agonist stimulation. The corrected labeled protein intensities were normalized to a starting value of one and then averaged with values from multiple cells.

RESULTS

Production of a Functional Fluorescent G Protein αs Subunit—Because the amino and carboxyl termini of G protein α subunits are important for interactions with receptors, effectors, the G protein βγ subunits, and the plasma membrane (38–40), we attempted to produce a functional αs-GFP by inserting GFP into αs at the position corresponding to an insertion site that produced a functional αs-GFP (41), but this fusion protein did not have activity (data not shown). We then used a random insertion approach that utilized a synthetic transposon containing GFP within the TrpC transposon. Surprisingly, the most functional fluorescent fusion protein obtained contained the GFP within the αA helix of αs whereas several insertions in exposed loops were not functional. This functional fusion protein was not expressed as well as αs was, but, when corrected for expression level, had comparable activity to that of αs (42).

In parallel with the random insertion approach, we produced an αs-CFP fusion (Fig. 1A), based on a report that inserting GFP in the α1/αA loop, the site of alternative splicing in αs, leaves function intact (20). αs-CFP differs from the previously published αs-GFP construct in that it contains a Ser-Gly-Gly-Gly-Gly-Ser linker on each side of the inserted CFP. We found that such a linker was essential for producing an αs-GFP fusion that had the same expression and activity levels as those of αs (41). The expression levels (Fig. 1B) and activities (Fig. 1C) of αs and αs-CFP were similar, and αs-CFP produced a fluorescent signal in the plasma membranes of HEK-293 cells (Fig. 2). As with αs-GFP, the Ser-Gly-Gly-Gly-Gly-Ser linkers appear to be important for activity, because the activity of αs-CFP was greater than that of a comparable αs-GFP fusion lacking these linkers (20) (Fig. 1D), although the expression levels of the two fusion proteins were similar (data not shown).

Production of a Functional Fluorescent G Protein β1γ2 Complex—We applied the strategy of BiFC (22) to visualize β1γ2 dimers (23). BiFC involves the production of a fluorescent signal by two nonfluorescent fragments of YFP when they are brought together by interactions between proteins fused to each fragment. Briefly, a functional fluorescent β1γ2 dimer was produced by fusing an amino-terminal YFP fragment (residues 1–158, referred to as YFP-N) to the amino terminus of β1 and a carboxyl-terminal YFP fragment (residues 159–238, referred to as YFP-C) to the amino terminus of γ2. When expressed together, YFP-N-β1 and YFP-C-γ2 produced a fluorescent signal in the plasma membrane (Fig. 2) that was not seen when either subunit was expressed alone (23). Functionality of YFP-N-β1/YFP-C-γ2 complexes was demonstrated by the ability to

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**Fig. 3.** Quantification of stimulus-dependent changes in the subcellular localization patterns of αs-CFP and YFP-N-β1/YFP-C-γ2. In this and all subsequent figures, cells were stimulated with 10 μM isoproterenol immediately after time = 0. A, stimulus-dependent loss of αs-CFP (filled circles, 18 cells) and YFP-N-β1/YFP-C-γ2 (filled squares, 18 cells) intensity from the plasma membrane. In unstimulated cells, the intensities of αs-CFP (open circles, 18 cells) and YFP-N-β1/YFP-C-γ2 (open squares, 20 cells) in the plasma membrane did not change. Measurements of αs-CFP were made in cells that co-expressed αs-CFP, YFP-Mem, and unlabeled β1, γ2, and β2AR. Measurements of YFP-N-β1/YFP-C-γ2 were made in cells that co-expressed YFP-N-β1/YFP-C-γ2, CFP-Mem, and unlabeled αs and β2AR. Values are the mean ± S.E. B, stimulus-dependent increases in intensity of αs-CFP (circles) and YFP-N-β1/YFP-C-γ2 (squares) in the cytoplasm (excluding labeled vesicles and the nucleus) in cells expressing αs-CFP, YFP-N-β1/YFP-C-γ2, β2AR, and mRFP (cytoplasm marker). Values represent the mean ± S.E. of 22 cells. C, stimulus-dependent labeling of vesicles with αs-CFP and YFP-N-β1/YFP-C-γ2. Areas of vesicles labeled with αs-CFP (circles), YFP-N-β1/YFP-C-γ2 (squares), and both αs-CFP and YFP-N-β1/YFP-C-γ2 (triangles) are shown. Values represent the mean ± S.E. of the same 22 cells that were analyzed for cytoplasm responses in B.
potentiate activation of adenylyl cyclase by αs in COS-7 cells (23). The BiFC method assures exclusive visualization of β2-AR dimers rather than individual subunits and leads to more selective labeling of the plasma membrane than is obtained with individually tagged subunits, which when co-expressed, are not functional (23).

Stimulation of the β2-AR Leads to Internalization of Both αs and βγγγ Subunits and Substantial Co-localization of These Gs Subunits on Vesicles—To visualize and investigate the regulation of the subcellular localization patterns of the subunits of Gs during signaling, we imaged αs-CFP and YFP-N-βγγγ and subunits of Gs after agonist stimulation in transfected HEK-293 cells. Stimulation of cells co-expressing αs-CFP, YFP-N-βγγγ, and the β2-AR with the β2-adrenergic agonist, isoproterenol, resulted in internalization of both αs-CFP (red in Fig. 2) and YFP-N-βγγγ (green in Fig. 2) from the plasma membrane. Initially, upon leaving the plasma membrane, αs-CFP and YFP-N-βγγγ exhibited a diffuse staining pattern in the cytoplasm (Fig. 2, 3 min). Subsequently, the signals for these Gs subunits exhibited significant co-localization (in yellow) on intracellular vesicles (Fig. 2, 24 min).

To quantify the distribution and movements of the labeled proteins following stimulation, we developed image analysis protocols to measure the intensity, area, and overlap of labeled cellular features. The pixels in each time course image that corresponded to a specific cellular feature such as the plasma membrane or intracellular vesicles were marked or segmented and analyzed as described under “Experimental Procedures.” The amounts of αs-CFP and YFP-N-βγγγ signal that moved out of the plasma membrane upon stimulation were quantified in cells co-expressing αs-CFP or YFP-N-βγγγ and a plasma membrane marker (YFP-Mem or CFP-
Isoproterenol-stimulated internalization of $\beta_2$AR-GFP, but not $\alpha_2$-CFP or YFP-N-$\beta_1$/YFP-C-$\gamma_7$, is inhibited by Dyn1(K44A)-mRFP. Top rows, before stimulation; bottom rows, after stimulation. In cells transfected with Dyn1(K44A)-mRFP, expression was confirmed by its fluorescence in each of the analyzed cells. A and B, Dyn1(K44A)-mRFP blocks isoproterenol-stimulated internalization of $\beta_2$AR-GFP. Cells were...
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Mem) to identify plasma membrane pixels and to control for changes in cell shape during the responses. The plasma membrane signals for \( \alpha_C \)-CFP (Fig. 3A, filled circles) and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) (Fig. 3A, filled squares) decreased with the same kinetics and the responses were essentially complete by 6 min. In the absence of stimulation, changes in the plasma membrane intensity of \( \alpha_C \)-CFP (Fig. 3A, open circles) and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) (Fig. 3A, open squares) did not occur.

The amounts of \( \alpha_C \)-CFP and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) signal that stained the cytoplasm diffusely were quantified relative to that of a cytoplasm marker (mRFP) and were determined for intracellular pixels, excluding vesicles that became labeled and the nucleus (see “Experimental Procedures”). This analysis demonstrated that the cytoplasm signal for both \( \alpha_C \)-CFP (Fig. 3B, circles) and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) (Fig. 3B, squares) increased upon stimulation at rates similar to that at which they decreased in the plasma membrane. Labeling of vesicles with \( \alpha_C \)-CFP and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) was quantified by determining the per cell areas of the labeled vesicles and the extent to which vesicles labeled with the two signals overlapped (Fig. 3C). The total areas of vesicles labeled with either \( \alpha_C \)-CFP (Fig. 3C, circles) or YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) (Fig. 3C, squares) increased with the same kinetics and to the same extent. Approximately 50% of the vesicle areas contained both signals (Fig. 3C, triangles). This is an underestimate of the overlap of \( \alpha_C \)-CFP and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) on vesicles, because vesicle movement in between acquisition of the sequential images reduced the measured overlap.

Stimulus-dependent decreases in the plasma membrane intensity of \( \alpha_C \)-CFP were somewhat greater in magnitude than those of YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) (Fig. 3A) and the increases in cytoplasm signal were greater for \( \alpha_C \)-CFP than for YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) (Fig. 3B). However, because the stimulus-dependent changes in the intensities of the two signals in these locations were determined relative to each of their starting values and do not represent absolute amounts of the proteins, these changes in the intensities of \( \alpha_C \)-CFP and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) are not directly comparable. Nevertheless, the similar timing of the \( \alpha_C \)-CFP and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) internalization responses (Fig. 3) and the similar localization patterns of these \( G \) protein subunits in the diffuse cytoplasm and on vesicles (Fig. 2) suggest that the activated populations may traffic together.

**Vesicles Labeled with \( \alpha_C \) and \( \beta_1 \gamma_7 \) Exhibit Minimal Overlap with \( \beta_2 \)AR-labeled Vesicles**—The trafficking patterns of the \( G \) subunits were compared directly with that of the \( \beta_2 \)AR in cells that co-expressed the labeled proteins. In cells co-expressing \( \alpha_C \)-CFP, unlabeled \( \beta_1 \gamma_7 \), and \( \beta_2 \)AR-GFP, there was minimal overlap between vesicles labeled with \( \alpha_C \)-CFP (red in Fig. 4A) and those labeled with \( \beta_2 \)AR-GFP (green in Fig. 4A). Image analysis of these cells demonstrated that similar amounts of vesicles, as defined by their per cell areas, became labeled with \( \alpha_C \)-CFP (Fig. 4B, filled circles) and \( \beta_2 \)AR-GFP (Fig. 4B, open circles), despite the minimal overlap of the two vesicle populations (Fig. 4B, filled triangles). Similarly, in cells that co-expressed unlabeled \( \alpha_s \), YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \), and \( \beta_2 \)AR-CFP, there was minimal overlap between vesicles labeled with YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) (red in Fig. 4C) and those labeled with \( \beta_2 \)AR-CFP (green in Fig. 4C). Again, image analysis showed that similar amounts of vesicles became labeled with YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) (Fig. 4D, filled circles) and \( \beta_2 \)AR-CFP (Fig. 4D, open circles), although the two vesicle populations did not co-localize (Fig. 4D, filled triangles). In addition, the fluorescent \( G \) subunit vesicles (filled circles in Fig. 4, B and D) labeled vesicles more rapidly than the fluorescent \( \beta_2 \)AR (open circles in Fig. 4, B and D).

**Receptor-stimulated Internalization of the \( \beta_2 \)AR, but Not \( \alpha_C \) or \( \beta_1 \gamma_7 \)** is Inhibited by a Dominant Negative \( G \) Mutant—The minimal overlap of \( \alpha_C \)-CFP and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) vesicles with \( \beta_2 \)AR-labeled vesicles suggested that the internalization pathways of these \( G \) subunits might differ from that of the \( \beta_2 \)AR. To test this hypothesis, we determined the effects on \( G \) subunit and \( \beta_2 \)AR internalization of a dominant negative \( G \) mutant fused to mRFP, Dyn1(K44A)-mRFP. The amounts of \( \beta_2 \)AR-GFP, \( \alpha_C \)-CFP, and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) in the plasma membrane (relative to CFP-Mem or YFP-Mem) before and after stimulation with isoproterenol were determined in the presence and absence of Dyn1(K44A)-mRFP. Dyn1(K44A)-mRFP effectively blocked internalization of \( \beta_2 \)AR-GFP (Fig. 5, A, B, and G), confirming previous studies (43, 44). However, Dyn1(K44A)-mRFP did not reduce either the magnitude or the frequency of the internalization responses of \( \alpha_C \)-CFP (Fig. 5, C, D, and H) or YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) (Fig. 5, E, F, and I).

**Vesicles Labeled with the \( \beta_2 \)AR Exhibit Greater Co-localization with RhoB-labeled Endosomes Than Do Vesicles Labeled with \( \alpha_C \) or \( \beta_1 \gamma_7 \)**—The initial diffuse labeling of the cytosol by both \( \alpha_C \)-CFP and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) after stimulation and the inability of Dyn1(K44A)-mRFP to block their internalization from the plasma membrane suggested that these \( G \) subunits may not directly internalize on vesicles. However, the subsequent labeling of vesicles by both \( \alpha_C \)-CFP and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) suggested that vesicular trafficking may play a role in effector modulation and/or the recycling of \( G \) back to the plasma membrane. Although \( G \) subunit-labeled vesicles did not co-localize well with \( \beta_2 \)AR-labeled vesicles (Fig. 4), we investigated the possibility that the \( G \) subunit-labeled vesicles might be endosomes, because diverse internalization pathways can converge on endosomes (45).

To explore the nature of the vesicles labeled by the \( G \) subunits, stimulus-dependent overlap of the \( G \) subunits and the \( \beta_2 \)AR with RhoB-labeled endosomes was measured. RhoB has been reported to associate with early endosomes (34) and multivesicular bodies, a prelysosomal compartment thought to be involved in sorting internalized receptors for degradation (35). Vesicles labeled with \( \beta_2 \)AR-GFP that formed after stimulation occupied similar per cell areas as did CFP-RhoB-labeled endosomes, which exhibited unchanged areas upon stimulation (Fig. 6, A and B). After stimulation, ~50% of the RhoB-labeled vesicle area overlapped with that of \( \beta_2 \)AR-labeled vesicles (Fig. 6, C and D). Cells were transfected with \( \alpha_C \)-CFP, YFP-Mem, and either mRFP (A) or Dyn1(K44A)-mRFP (B). C and D, Dyn1(K44A)-mRFP does not block isoproterenol-stimulated internalization of \( \alpha_C \)-CFP. Cells were transfected with \( \alpha_C \)-CFP, \( \beta_1 \gamma_7 \), \( \beta_2 \)AR-GFP, YFP-Mem, and either mRFP (E) or Dyn1(K44A)-mRFP (F). In the segments image, the cell border is gray, the segmented plasma membrane is green, the portion of the plasma membrane segment used for analysis of intensity is red, and intracellular vesicles labeled with \( \beta_2 \)AR-GFP, \( \alpha_C \)-CFP, or YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) are yellow. Bars = 10 \( \mu \)m. Videos for A–F of this figure are available under Supplemental Materials. G–I, quantification of internalization responses of \( \beta_2 \)AR-GFP, \( \alpha_C \)-CFP, and YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) relative to the plasma membrane marker (CFP-Mem or YFP-Mem) in the presence and absence of Dyn1(K44A)-mRFP. The number of cells analyzed and the percentage of cells that exhibited detectable responses (see “Experimental Procedures”) are listed below. G, values for \( \beta_2 \)AR-GFP in the absence of Dyn1(K44A)-mRFP (filled circles, 30 cells, 87% responding) and presence of Dyn1(K44A)-mRFP (open circles, 38 cells, 28% responding). H, values for \( \alpha_C \)-CFP in the absence of Dyn1(K44A)-mRFP (filled circles, 22 cells, 73% responding) and the presence of Dyn1(K44A)-mRFP (open circles, 30 cells, 77% responding). I, values for YFP-N-\( \beta_1 \)/YFP-C-\( \gamma_7 \) in the absence of Dyn1(K44A)-mRFP (filled circles, 30 cells, 60% responding) and in the presence of Dyn1(K44A)-mRFP (open circles, 39 cells, 72% responding). Values represent mean ± S.E. YN indicates YFP-N and YC indicates YFP-C.
FIG. 6. β₂AR-GFP exhibits a greater amount of overlap with RhoB-labeled endosomes than do α₅-CFP and YFP-N-β₁/YFP-C-γ₁. A, β₂AR-GFP (red) exhibits significant overlap (yellow) with RhoB-labeled endosomes (green) after stimulation. Cells were transfected with α₅, β₁, γ₁, β₂AR-GFP, CFP-RhoB, and mRFP. Top row, before stimulation; bottom row, 25 min after stimulation. Cell border is blue. Bar = 10 μm. B, quantification of stimulus-dependent labeling of vesicles with β₂AR-GFP and CFP-RhoB. Areas of vesicles labeled with β₂AR-GFP (filled circles), CFP-RhoB (open circles), and both β₂AR-GFP and CFP-RhoB (filled triangles) are shown. Values represent the mean ± S.E. from 22 cells. C, α₅-CFP (red) exhibits partial overlap (yellow) with RhoB-labeled endosomes (green) after stimulation. Cells were transfected with α₅-CFP, β₁, γ₁, β₂AR, YFP-RhoB, and mRFP. Top row, before stimulation; bottom row, 26 min after stimulation. Bar = 10 μm. D, quantification of stimulus-dependent labeling of vesicles with α₅-CFP and YFP-RhoB. Areas of vesicles labeled with α₅-CFP (filled circles), YFP-RhoB (open circles), and both α₅-CFP and YFP-RhoB (filled triangles) are shown. Values represent the mean ± S.E. from 22 cells. E, YFP-N-β₁/YFP-C-γ₁ (red) exhibits partial
Vesicles Labeled with αs and βγy Exhibit Greater Co-localization with Rab11-labeled Endosomes Than Do Vesicles Labeled with the Rab11-labeled vesicles than the RhoB-labeled vesicles co-localized with only ~25% of the Rab11-labeled endosome area (Fig. 6, C–F). A previous study using immunohistochemistry demonstrated activation-dependent redistribution of αs to the cytosol in HEK-293 cells stably transfected with the βAR and HA-tagged αs (19). The staining pattern for internalized αs was punctate, but could be distinguished from that of the β2AR. In a study using a different αs-GFP construct, isoproterenol stimulation of COS-1 cells resulted in a partial decrease in association of αs-GFP with some regions of the plasma membrane as well as insertion of αs-GFP at other membrane sites (20). Differences between our results with αs-GFP expressed in HEK-293 cells and these previous results with αs-GFP could be because of the greater activity of the αs-CFP construct and/or the different cell types examined. Stimulus-dependent βγ internalization has not been imaged previously, but using an enzyme-linked immunosorbent assay, redistribution of βγ from the plasma membrane to low density microsomes was observed in response to isoproterenol stimulation (21).

Our results indicate that αs and the β2AR dissociate upon hormonal stimulation and utilize different mechanisms for internalization from the plasma membrane, because internalization of the β2AR, but not αs or βγy, can be blocked by a dominant negative dynamin 1 mutant. The inability of Dyn1(K44A)-mRFP to block internalization of αs extends a previous study in which hypertonic sucrose, which inhibits the formation of clathrin-coated pits, blocked internalization of the β2AR, but not αs (19). Because dynamin is involved in both clathrin-mediated endocytosis (52, 53) and endocytosis of caveolae (54), our results demonstrate that internalization of αs and βγy is independent of both of these mechanisms. However, because αs and βγy subsequently exhibit partial co-localization with Rabδ- and Rab11-labeled endosomes, these Gs subunits appear to join the endosomal pathway by a different and as yet uncharacterized mechanism. There is precedent for alternative pathways from the plasma membrane to endosomes in that endocytosis of the M2-muscarinic receptor is independent of clathrin, but involves subsequent transfer to endosomal compartments of the clathrin-dependent pathway (45). Our observation that both αs and βγy leave the plasma membrane upon activation is consistent with previous reports that the combined effects of their plasma membrane targeting motifs are important for targeting each of them to the plasma membrane (14, 55, 56). Activation-dependent depalmitoylation of αs is associated with its internalization from the plasma membrane to the cytoplasm (18, 19), and mutant forms of αs containing alternative plasma membrane-targeting motifs do not show activation-dependent internalization (57). It is possible that depalmitoylation of αs is sufficient to result in movement of βγ to the cytoplasm, based on the observation that efficient plasma membrane targeting of βγ2 expressed in HEK-293 cells required either co-expression of αs or introduction of a palmitoylation site into γ2 (14). Subsequent associa-

**DISCUSSION**

By imaging functional fluorescent fusion proteins in living cells, we have observed internalization of both the α and βγ components of the Gs heterotrimer, αsβγy, upon stimulation of the β2AR. αs-CFP, in which CFP was inserted into αs at an internal site, exhibited similar activity to that of αs, βγ, and γγ were imaged exclusively in the form of βγ complexes by means of BiFC (22), using fusion proteins, YFP-N-βγ and YFP-C-γγ, that only produced fluorescence and functional activity when expressed together (23). Initially, after leaving the plasma membrane, αs-CFP and YFP-N-βγ/C-γγ labeled the cytoplasm diffusely, with the same kinetics. Subsequently, they exhibited a high degree of co-localization on vesicles that exhibited minimal overlap with β2AR-labeled vesicles. In general, our results confirm previous imaging studies on the trafficking of αs. Moreover, to our knowledge, this study represents the first visualization of stimulus-dependent internalization of βγ. In addition, we recently developed methodologies enabled direct comparisons to be made between the trafficking patterns of αs and βγy and between these Gs subunits and the β2AR, to investigate potential regulatory mechanisms, and to address the issue of how the specificity of αs and βγ interactions is maintained during signaling cycles.

A previous study using immunohistochemistry demonstrated activation-dependent redistribution of αs to the cytosol in HEK-293 cells stably transfected with the β2AR and HA-tagged αs (19). Our results indicated that both αs-CFP and YFP-Rab11 (31) selectively labeled a small perinuclear region (Fig. 7, A, C, and E). Segmentation analysis demonstrated that the cellular area occupied by Rab11-labeled vesicles (open circles in Fig. 7) was much smaller than that occupied by Rho-B-labeled vesicles (open circles in Fig. 6). However, we observed significant stimulus-dependent overlap in the perinuclear region between αs-CFP and YFP-Rab11 (Fig. 7, A and B). Similar overlap was obtained with YFP-N-βγ/YFP-C-γγ and CFP-Rab11 (Fig. 7, C and D). Approximately 40% of the Rab11-labeled vesicles co-localized with Gs subunit-labeled vesicles by the ends of the time courses. The total area of overlap between Rab11-labeled vesicles and those labeled with Gs subunits (filled triangles in Fig. 7, B and D) was less than that between Rho-B- and Gs subunit-labeled vesicles (filled triangles in Fig. 6, D and F). However, a greater percentage of the Rab11-labeled vesicles than the Rho-B-labeled vesicles co-localized with Gs subunit-labeled vesicles. In contrast, there was minimal overlap between β2AR-GFP and CFP-Rab11 (Fig. 7, E and F), confirming previous studies showing that the β2AR recycles to the plasma membrane directly from early endosomes via a Rab4-dependent mechanism (51), rather than through the slow perinuclear recycling endosome route.

**FIGURE 7**

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6, A and B). As with αs-CFP and YFP-N-βγ/YFP-C-γγ vesicles (Figs. 2 and 3), the actual overlap was underestimated, because of the sequential acquisition of images. In contrast, although vesicles labeled with αs-CFP or YFP-N-βγ/YFP-C-γγ occupied similar per cell areas, respectively, as did YFP-Rho-B- and CFP-RhoB-labeled endosomes, the Gs subunit vesicles co-localized with only ~25% of the Rab11-labeled endosome area (Fig. 6, C–F).
FIG. 7. Vesicles labeled with αs-CFP and YFP-N-β1/YPF-C-γ7, but not β2AR-GFP exhibit partial co-localization with Rab11-labeled endosomes. A, αs-CFP (red) exhibits overlap (yellow) in the perinuclear region with YFP-Rab11 (green) after stimulation. Cells were transfected with αs-CFP, β1, γ7, β2AR, YFP-Rab11, and mRFP. Top row; before stimulation; bottom row; 24 min after stimulation. Cell border is blue. Bar = 10 μm. B, quantification of stimulus-dependent labeling of vesicles with αs-CFP and YFP-Rab11. Areas of vesicles labeled with αs-CFP (filled circles), YFP-Rab11 (open circles), and both αs-CFP and YFP-Rab11 (filled triangles) are shown. Values represent the mean ± S.E. from 20 cells. C, YFP-N-β1/ypF-C-γ7 (red) exhibits overlap (yellow) in the perinuclear region with CFP-Rab11 (green) after stimulation. Cells were transfected with αs, YFP-N-β1, YFP-C-γ7, β2AR, CFP-Rab11, and mRFP. Top row; before stimulation; bottom row; 28 min after stimulation. Bar = 10 μm. D, quantification of stimulus-dependent labeling of vesicles with YFP-N-β1/YPF-C-γ7 and CFP-Rab11. Areas of vesicles labeled with YFP-N-β1/YPF-C-γ7 (filled circles), CFP-Rab11 (open circles), and both YFP-N-β1/YPF-C-γ7 and CFP-Rab11 (filled triangles) are shown. Values represent the mean ± S.E. from 20 cells. E, β2AR-GFP (red) does not overlap with CFP-Rab11 (green) after stimulation. Cells were transfected with αs, β1, γ7, β2AR-GFP, CFP-Rab11, and mRFP. Top row; before stimulation; bottom row; 26 min after stimulation. Bar = 10 μm. F, quantification of stimulus-dependent labeling of vesicles with β2AR-GFP and CFP-Rab11. Areas of vesicles labeled with β2AR-GFP (filled circles), CFP-Rab11 (open circles), and both β2AR-GFP and CFP-Rab11 (filled triangles) are shown. Values represent mean ± S.E. of 20 cells. Videos for A, C, and E of this figure are available under Supplemental Materials. YN indicates YFP-N and YC indicates YFP-C.
tion of cytosolic αs and βγ with vesicles could be due to reassembly after its activation. Another potential contributing factor to the internalization of both αs and βγ could be their transient dissociation upon stimulation. Based on in vitro studies, the G protein α and βγ subunits have been thought to dissociate upon activation, but recent studies using fluorescence resonance energy transfer have produced differing results as to whether or not this actually takes place in vivo (58–60). Because each of these studies utilized a different G protein heterotrimer, it is possible that some, but not all G proteins dissociate upon activation. Future fluorescence resonance energy transfer studies will address the issue of whether or not the Gs subunits dissociate upon activation. In addition, it will also be interesting to determine whether βγ internalization requires internalization of the associated α subunit. For instance, when Gq is activated by stimulation of the αq-adrenergic receptor, αq does not internalize (41).

Localization of αs and β1γ2 on vesicles may be important for certain Gs effector functions. For instance, there is evidence that both the αs and βγ subunits of Gs may play roles in regulating the fusion of early endosomes (61, 62). In addition, the Gq subunits have been implicated in the regulation of transcytosis of the polymeric immunoglobulin receptor (63, 64), and activation of αq stimulates transport of influenza hemagglutinin protein from the trans-Golgi network to the apical surface of Madin-Darby canine kidney cells (65). Additional characterization of the vesicles labeled by αs and β1γ2 may help to elucidate the roles of these vesicles in vesicular trafficking.

There is clearly a population of vesicles labeled with both αs and β1γ2 that do not overlap with either Rhob or Rab11-labeled endosomes. Future experiments using other markers will attempt to identify these vesicles.

Localization of αs and β1γ2 to Rab11-labeled vesicles may indicate that these Gs subunits recycle to the plasma membrane via the slow perinuclear recycling endosome route, which Rab11 plays a role in regulating (24). Slow recycling of certain G protein-coupled receptors, such as the vasopressin V2, somatostin 3, and CXXC chemokine 2 receptors, is blocked by overexpression of dominant negative Rab11 mutants (51). We observed little overlap between vesicles labeled with the β2AR and Rab11, consistent with a previous study showing that the β2AR exhibits relatively little co-localization with Rab11 unless cells are treated with the proton pump inhibitor, fumitremorgin C (69), which raises endosome and lysosome pH and blocks the formation of vesicles that traffic between endosomes to lysosomes.

The high degree of co-localization of αs and β1γ2 on intraacellular vesicles and the overlap of some of these vesicles with Rab11-labeled endosomes may indicate a means of recycling specific αsβγ combinations to the plasma membrane. Although different βγ combinations generally exhibit similar abilities to modulate the activities of effectors such as adenyl cyclase (70), phospholipase C (71), and GIRQ channels (72), reconstitution experiments have indicated clear differences in the αβγ combinations that are preferred by particular receptors (73–78). Moreover, inactivation of specific G protein subunits in vivo using antisense (2–6) and ribozyme (7, 8) strategies has demonstrated a remarkable specificity of interaction between receptors, αβγ combinations, and effectors. Future studies will investigate whether αs and β1γ2 recycle to the plasma membrane together by following their localization patterns upon removal of the stimulus and test for a potential regulatory role of Rab11 in this process. In addition, we will investigate whether other αsβγ combina-
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45. Delaney, K. A., Murph, M. M., Brown, I. M., and Radhakrishna, H. (2002) J. Biol. Chem. 277, 33439–33446
46. Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G., and Tsien, R. Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6803–6808
47. Yamaguchi, N., and Pakuda, M. N. (1995) J. Biol. Chem. 270, 12170–12176
48. Marra, P., Maffucci, T., Daniele, T., Tullio, G. D., Ikehara, Y., Chan, E. K., Luini, A., Benzonoueneko, G., Mironov, A., and De Matteis, M. A. (2001) Nat. Cell. Biol. 3, 1101–1113
49. Barr, F. A., Nakamura, N., and Warren, G. (1998) EMBO J. 17, 3258–3268
50. Urbe, S., Huber, L. A., Zerial, M., Touze, S. A., and Parton, R. G. (1993) FEBS Lett. 334, 175–182
51. Seachrist, J. L., Anborgh, P. H., and Ferguson, S. S. (2000) J. Biol. Chem. 275, 27221–27228
52. Herskovits, J. S., Burgess, C. C., Obar, R. A., and Vallee, R. B. (1993) J. Cell Biol. 122, 565–578
53. van der Bliek, A. M., Redelmeier, T. E., Damke, H., Tisdale, E. J., Meyerowitz, E. M., and Schmid, S. L. (1993) J. Cell Biol. 122, 553–563
54. Henley, J. R., Krueger, E. W., Oswald, B. J., and McNiven, M. A. (1998) J. Cell Biol. 141, 85–99
55. Evanko, D. S., Thiyagarajan, M. M., Siderovski, D. P., and Wedegaertner, P. B. (2001) J. Biol. Chem. 276, 23945–23953
56. Michaelson, D., Ahearn, I., Bergo, M., Young, S., and Philips, M. (2002) Mol. Biol. Cell 13, 3294–3302
57. Thiyagarajan, M. M., Biggas, E., Van Tol, H. H., Hebert, T. E., Evanko, D. S., and Wedegaertner, P. B. (2002) Biochemistry 41, 9470–9484
58. Janetopoulos, C., Jin, T., and Devreotes, P. (2001) Science 291, 2408–2411
59. Buzeman, M., Frank, M., and Lohse, M. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 16077–16082
60. Yi, T. M., Kitano, H., and Simon, M. I. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10764–10769
61. Haraguchi, L., and Rodbell, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5964–5968
62. Colombo, M. I., Mayorga, L. S., Nishimoto, I., Ross, E. M., and Stahl, P. D. (1994) J. Biol. Chem. 269, 14919–14923
63. Bomsel, M., and Moster, K. E. (1993) J. Biol. Chem. 268, 25824–25835
64. Barroso, M., and Setul, E. S. (1994) J. Cell Biol. 124, 83–100
65. Pimplikar, S. W., and Simons, K. (1993) Nature 362, 456–458
66. Inamura, G., Le Gouill, C., Balamontis, M., and Birnbaumer, M. (2001) J. Biol. Chem. 276, 13096–13103
67. Kreuzer, O. J., Krisch, B., Dery, O., Bunnett, N. W., and Meyerhof, W. (2001) J. Neuroendocrinol. 13, 279–287
68. Fan, G. H., Lapierre, L. A., Goldenring, J. R., and Richmond, A. (2003) Blood 101, 2115–2124
69. Moore, R. H., Tuffaha, A., Millman, E. E., Dai, W., Hall, H. S., Dickey, B. F., and Knoll, B. J. (1999) J. Cell Sci. 112, 329–338
70. Iniguez-Lhuill, J. A., Simon, M. I., Robishaw, J. D., and Gilman, A. G. (1992) J. Biol. Chem. 267, 23409–23417
71. Ueda, N., Iniguez-Lhuill, J. A., Lee, A. V., Robishaw, J. D., and Gilman, A. G. (1994) J. Biol. Chem. 269, 4388–4395
72. Mirshahi, T., Robillard, L., Zhang, H., Hebert, T. E., and Logothetis, D. E. (2002) J. Biol. Chem. 277, 7348–7355
73. Richardson, M., and Robishaw, J. D. (1999) J. Biol. Chem. 274, 13525–13533
74. Figler, R. A., Lindorfer, M. A., Graber, S. G., Garrison, J. C., and Linden, J. (1997) Biochemistry 36, 16288–16299
75. Buskens, C. A., Zheng, Y., Hallak, H., Graham, T. E., Miller, H. A., Murr, K. D., Molinos, P. B., and Manning, D. R. (1995) J. Biol. Chem. 270, 18691–18699
76. Hou, Y., Aspiazu, I., Smrcka, A., and Gautam, N. (2000) J. Biol. Chem. 275, 38961–38964
77. Hou, Y., Chang, V., Capper, A. B., Tassell, R., and Gautam, N. (2001) J. Biol. Chem. 276, 19982–19988
78. McIntyre, W. E., MacCleery, G., and Garrison, J. C. (2001) J. Biol. Chem. 276, 15981–15989
79. Ormo, M., Cubbitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996) Science 273, 1392–1396
80. Tesmer, J. J., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997) Science 278, 1807–1916
81. Guyer, C. A., Horstman, D. A., Wilson, A. L., Clark, J. D., Cragoe, E. J., and Limbird, L. E. (1990) J. Biol. Chem. 265, 17367–17377