A β-Arrestin/Green Fluorescent Protein Biosensor for Detecting G Protein-coupled Receptor Activation*

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G protein-coupled receptors (GPCR) represent the single most important drug targets for medical therapy, and information from genome sequencing and genomic data bases has substantially accelerated their discovery. The lack of a systematic approach either to identify the function of a new GPCR or to associate it with a cognate ligand has added to the growing number of orphan receptors. In this work we provide a novel approach to recognize the activation of pharmacologically distinct GPCRs. This approach relies on the use of β-arrestin/GFP, a single cell based assay to monitor GPCR activation and GPCR-arrestin interactions. Confocal microscopy demonstrates the translocation of βarr2-GFP to more than 15 different ligand-activated GPCRs. These data clearly support the common hypothesis that the β-arrestin binding of an activated receptor is a convergent step of GPCR signaling, increase by 5-fold the number of GPCRs known to interact with β-arrestins, demonstrate that the cytosol is the predominant reservoir of biologically active β-arrestins, and provide the first direct demonstration of the critical importance of G protein-coupled receptor kinase phosphorylation to the biological regulation of β-arrestin activity and GPCR signal transduction in living cells. The use of βarr2-GFP as a biosensor to recognize the activation of pharmacologically distinct GPCRs should accelerate the identification of orphan receptors and permit the optical study of their signal transduction biology intractable to ordinary biochemical methods.

The G protein-coupled receptor (GPCR)1 superfamily is growing rapidly (1–3), creating many new orphan receptors whose properties remain undefined (4–6).2 Currently characterized GPCRs display many distinct pharmacologies. For example, they interact with a vast array of ligands and generate intracellular signals by multiple second messenger pathways (4, 8, 9). Based on work with rhodopsin and the β2-adrenergic receptor (β2AR), it has been postulated that members of the GPCR superfamily desensitize via a common mechanism involving the arresting proteins visual arrestin, β-arrestin1 and β-arrestin2 (10–13). However, mainly due to the inherent difficulties of examining the interaction of the components mediating desensitization in their native environment or the need for purified reconstituted systems, this has not been clearly established for many GPCRs. Biochemical studies indicate that arrestins regulate GPCR signal transduction (desensitization) by binding agonist-activated receptors that have been phosphorylated by G protein-coupled receptor kinases (GRKs) (12). While the functional source of arrestin molecules targeted to receptors remains unknown, it is apparent that arrestin binding terminates signaling by interdicting receptor interaction with G proteins (12).

To characterize the interaction between β-arrestin and different GPCRs and to assess the contribution of GRKs to this process, we examined using confocal microscopy how a green fluorescent protein/β-arrestin2 conjugate responded to ligand-mediated receptor activation. The results demonstrate a critical role for GRKs in the apparently universal regulation of GPCRs by β-arrestins. Moreover, they provide the first real-time, live-cell demonstration of a GPCR interacting with one of its regulatory proteins and demonstrate a practical role for βarr2-GFP in the study of GPCR activation.

EXPERIMENTAL PROCEDURES

Materials—Isoproterenol was obtained from Sigma and Research Biochemicals International. Anti-mouse antibody was obtained from Sigma and Molecular Probes. Mouse monoclonal antibody against the 12CA5 epitope was purchased from Boehringer Mannheim. Cell culture medium was obtained from Mediatech and fetal bovine serum from Atlanta Biologicals. Physiological buffers were from Life Technologies, Inc. Restriction enzymes were obtained from Promega or New England Biolabs, T4 ligase was from Promega, and Hot Tub DNA polymerase from Amersham. Plasmid containing variants of green fluorescent protein and anti-GFP antibodies were from CLONTECH.

Plasmid Construction, βarr2-GFP—Oligonucleotide primers surrounding the Xhol restriction site and C-terminal stop codon of β-arrestin2, in the expression vector pCMV5, were used to replace the stop codon with an in frame BamHI restriction site by directed mutagenesis (14). The product nucleotide was purified by electrophoresis on a 1.5% agarose gel and isolated. It was digested using XhoI and BamHI restriction enzymes, and the nucleotide fragment flanked by these sites was repurified. The N-terminal, proximal cDNA fragment of β-arrestin2 flanked by SacI/XhoI restriction sites was removed by digestion from pCMV5. It was ligated with the XhoI/BamHI fragment isolated above and with the purified expression vector (pS65T-GFP) that had been opened between the SacI/BamHI polylinker restriction sites using the respective enzymes (15). The resulting construct was grown in competent Escherichia coli, isolated, and verified by sequencing.

Cell Culture and Transfection—HEK-293 and COS cells were maintained and transfected as described previously (15, 16). Cells containing both receptor and β-arrestin constructs were transfected with between 5 and 10 μg of receptor cDNA in pcDNA/AMP and 0.5–1 μg of βarr2-β2AR, desensitization by binding agonist-activated receptors that have been phosphorylated by G protein-coupled receptor kinases (GRKs) (12).

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RESULTS

GFP because of its inherent fluorescence, represents a valuable biological reporter molecule for the study of GPCR signal transduction events (15, 18–21). The βarr2-GFP fusion protein (Fig. 1A), which is approximately 50% larger than β-arrestin2 and migrates more slowly on SDS-polyacrylamide gel electrophoresis (Fig. 1B), still retains its biological activity with respect to facilitating β2AR sequestration (22). In the absence of supplemental β-arrestins, the β2AR normally sequesters poorly in COS-7 cells (16). βarr2-GFP overexpression increases sequestration to the same extent as wild type β-arrestin2 (Fig. 1C).

Confocal microscopy of βarr2-GFP in an HEK-293 cell (Fig. 2A) shows that in the absence of receptor activation β-arrestins are not predominantly compartmentalized at the plasma membrane prior to agonist stimulation. Upon the addition of saturating concentrations of the agonist isoproterenol to the cell medium, an enhancement of plasma membrane fluorescence is observed with a concomitant loss of cytosolic fluorescence (Fig. 2B). This observation indicates that β-arrestins are not discretely compartmentalized within the cell. Moreover, the data demonstrate that β-arrestins are distributed throughout the cell cytoplasm and excluded from the nucleus (Fig. 2C). This observation is in contrast to the concave trace obtained after isoproterenol treatment, (D), which reflects the increased concentration of βarr2-GFP at the cell margins and the reduction of βarr2-GFP concentration in the cell cytoplasm that results from GPCR activation by agonist. Bar = 10 μm.

FIG. 1. Linear model of the β-arrestin2/S67T-GFP conjugate and its characterization by SDS-polyacrylamide gel electrophoresis and β2AR sequestration. A, the model indicates the relative size of β-arrestin2 compared with GFP. In this construct the C-terminal stop codon of β-arrestin2 is replaced by a BamHI restriction site, and β-arrestin2 cDNA is inserted in frame to GFP between SacI and BamHI. B, shown are Western blots of homogenates from HEK-293 cells expressing βarr2-GFP. Equal amounts of material were loaded into each lane. The gel on the left was exposed to anti-β-arrestin antibody (16), whereas the right gel was exposed to a mouse monoclonal antibody against GFP. The position of endogenous cellular β-arrestin2 is indicated by the lower band on the left. The heavy band below 71,000 (upper left) corresponding to βarr2-GFP is mirrored by a similar band in the right gel. In contrast, no band corresponding to endogenous cellular β-arrestin2 is observed with anti-GFP antibody exposure. C, β-arrestin activity can indirectly be assessed by measuring its effect on receptor sequestration (16, 22). The sequestration of β2AR in COS cells with and without overexpressed β-arrestin2 (left bars) or with and without overexpressed βarr2-GFP (rightmost bars) is presented. Wild type β-arrestin2 and βarr2-GFP enhance β2AR sequestration equally well, producing a 2.5- and 2.4-fold increase, respectively. (Results are average ± S.D. from triplicate experiments.)

Fig. 2. Confocal Microscopy of βarr2-GFP translocation in cells containing the β2AR. HEK-293 cells were transfected with 10 μg of plasmid cDNA containing the β2AR and 0.5–1.0 μg for βarr2-GFP. A and B, βarr2-GFP distribution is initially cytosolic with no significant nuclear or membrane enhancement apparent. C and D, following a 10 min stimulation with 20 μM isoproterenol, the real-time agonist-mediated redistribution of βarr2-GFP from the cytosol to the membrane is shown. The relative magnitudes of the βarr2-GFP concentrations can be quantified (A) before and (C) after agonist treatment. βarr2-GFP concentrations along a linear slice (blue line) made through the lower portion of the above cell in A and C are profiled in B and D, respectively. The flatness of the central portion of the trace in B corresponds to the uniform cytoplasmic distribution of βarr2-GFP prior to agonist treatment. However, in regions lacking βarr2-GFP, i.e. outside the cell and in the nucleus, the magnitude of the trace decreases. In contrast, the concave trace obtained after isoproterenol treatment, (D), reflects the increased concentration of βarr2-GFP at the cell margins and the reduction of βarr2-GFP concentration in the cell cytoplasm that results from GPCR activation by agonist. Bar = 10 μm.

Confocal microscopy of βarr2-GFP in an HEK-293 cell (Fig. 2A) shows that in the absence of receptor activation β-arrestins are distributed throughout the cytosol and excluded from the nucleus. Moreover, the data demonstrate that β-arrestins are not predominantly compartmentalized at the plasma membrane prior to agonist stimulation. Upon the addition of saturating concentrations of the agonist isoproterenol to the cell medium, an enhancement of plasma membrane fluorescence and a concomitant loss of cytosolic fluorescence (Fig. 2B) can be readily observed and quantified (Figs. 2, C and D). This observation indicates that β-arrestins are not discretely compartmentalized and that the entire cytoplasmic content represents a functional β-arrestin reservoir.

HEK-293 cells overexpressing the β2AR were used to investigate whether the main target of translocated βarr2-GFP was a plasma membrane site other than a GPCR. N-terminal epitope-tagged β2AR were cross-linked to one another prior to agonist exposure using a mouse monoclonal antibody against the epitope and a secondary goat-anti-mouse antibody conjugated to the fluorophore Texas Red. Fig. 3 demonstrates that the geometry of the agonist-induced time-dependent translocation of βarr2-GFP to the plasma membrane mirrors the distribution of preaggregated β2ARs, strongly suggesting that the primary targeted site of β-arrestin is the β2AR.

βarr2-GFP translocation to β2ARs is not limited to HEK-293
bars left

GPCRs immediately following agonist stimulation and GRK D hanced in the presence of overexpressed GRK2 (Fig. 6

membrane, 12CA5 (hemagglutinin)-tagged bAR were evaluated for their ability to mediate the movement of limited to the

D receptor for bAR, other members of the GPCR superfamily -arrestin (22). To further characterize the role of GRK phosphorylation in b-arrestin translocation, we examined the ability of a GRK phosphorylation-impaired mu tant Y326A-bAR to support b-arrestin redistribution (11, 17, 23, 24). Consistent with its inability to be phosphorylated by endogenous GRKs, the Y326A-b2AR mutant did not induce bAR-mediated translocation, only accurately monitors the biology of the GPCR activation process but the GPCR phosphorylation state as well.

To establish that agonist-induced barr2-GFP translocation represents a general property of GPCR activation and is not limited to the b2AR, other members of the GPCR superfamily were evaluated for their ability to mediate the movement of barr2-GFP in HEK-293 cells. Shown in Fig. 6 are results with the dopamine D1A receptor. Its behavior is representative of 16 different GPCRs that were tested belonging to the the angio tensin, a- and b-adrenergic, dopamine, endothelin, intestinal peptide, chemokine, and opioid receptor subfamilies. Activation of the D1A receptor with 20 μM dopamine produced an increase in the amount of membrane-associated barr2-GFP. Moreover, the increase in barr2-GFP translocation was enhanced in the presence of overexpressed GRK2 (Fig. 6D).

FIG. 3. Time course of barr2-GFP redistribution to plasma membrane, 12CA5 (hemagglutinin)-tagged bAR in HEK-293 cells as observed by confocal microscopy. To demonstrate that bARs were the target of intracellular barr2-GFP, receptors were reorganized into plasma membrane clusters (A) by cross-linking with a primary layer of monocolonal anti-epitope antibody followed by a secondary layer of Texas Red-conjugated goat anti-mouse antibody. The agonist-mediated time course of barr2-GFP redistribution to these receptors at 0, 3, and 10 min (B, panels left to right) is shown to correspond to the aggregated receptor distribution in (A, corresponding panels left to right). Arrows indicate some of the regions of colocalization. Bar = 10 μm.

FIG. 4. Influence of overexpressed GRK on redistribution of barr2-GFP in COS-7 cells. A–D, COS-7 cells were transiently transfected with barr2-GFP and bAR as described, and the agonist-mediated redistribution of barr2-GFP in the absence (A, C) or presence (B, D) of overexpressed GRK2 was determined. Both the rate and extent of agonist-mediated barr2-GFP translocation are observed to be enhanced by the overexpression of GRKs, and this is reflected by an increase in contrast in the photomicrographs between cytoplasm and membrane barr2-GFP fluorescence for the GRK untreated pair (A, C) and the GRK-treated pair (B, D). Bar = 10 μm.

FIG. 5. Influence of overexpressed GRK on redistribution of barr2-GFP to the Y326A-bAR in HEK-293 cells. The effect of overexpressed GRK2 on the agonist-mediated interaction between the phosphorylation-impaired Y326A-bAR and barr2-GFP in HEK-293 cells is shown. Cells without (A) and with (B) overexpressed GRK2 were exposed to agonist. barr2-GFP translocation in cells containing overexpressed GRK2 is more robust, as demonstrated (B), indicating an increased affinity of barr2-GFP for receptor. Bar = 10 μm.

Discussion

In this work we demonstrate that b-arrestin interacts with GPCRs immediately following agonist stimulation and GRK phosphorylation. barr2-GFP translocation was observed in response to more than 15 different GPCRs. Even though these GPCRs respond to a diverse array of ligands and different classes of G proteins, activation of each of the GPCRs elicits the agonist-dependent translocation of barr2-GFP, with the magnitudes of plasma membrane fluorescence signals ranging up to 10–20-fold above the intracellular background. While b-arrestin behavior is regulated by multiple components of the signal transduction system, it is particularly sensitive to how well cellular GRKs are able to phosphorylate a particular GPCR. This was demonstrated with both GRK2 and GRK5 (data not shown). For instance, following overexpression of GRK2 to force phosphorylation of the Y326A-bAR mutant, the mutant-mediated barr2-GFP translocation is indistinguishable from the wild type bAR-mediated response. Consequently,
with the appropriate cellular system, such as COS-7 cells in which endogenous GRKs and β-arrestins are relatively poorly expressed, the β-arrestin translocation paradigm could also be used to easily monitor the activity and specificity of each of the members of the GRK and arrestin families.

Biochemical measurements of GPCR properties, such as ligand binding, activation of G proteins or effectors, generation of second messengers, or extent of phosphorylation, assess functions that are receptor-specific and do not easily lend themselves to the development of rapid or convenient screening methods. However, since GPCR activation ultimately terminates with the association of β-arrestin and receptor, a convergent step of the GPCR signal transduction paradigm, the cellular visualization of the agonist-mediated translocation of βarr2-GFP provides a universal measure for detecting the activation of unknown GPCRs. Despite its present large size, the G protein-coupled receptor superfamily continues to expand rapidly as new receptors are discovered through automated sequencing of cDNA libraries. It is estimated that several thousand GPCRs may exist in the human genome, and at present with only a fraction of the genome sequenced, as many as 250 GPCRs have been cloned and only as few as 150 have been associated with ligands (4).² The means by which these or newly discovered orphan receptors will be associated with their cognate ligands and physiological functions represents a major challenge to biological and biomedical research. The identification of an orphan receptor generally requires an individualized assay and a guess as to its function. The interrogation of a GPCR’s signaling behavior by monitoring βarr2-GFP translocation eliminates these prerequisites, since it can be performed with unlabeled ligands and without any prior knowledge of other signaling events. It is sensitive, rapid, easily performed, and should be potentially applicable to nearly all GPCRs, since the majority of these receptors should desensitize by a common mechanism, i.e., interaction with β-arrestins.

The visualization of β-arrestin2 translocation represents the first direct real-time assessment in a living cell of the interaction of a GPCR with one of its regulatory components. Moreover, the rapid and profound increases in the relative and absolute amounts of plasma membrane-bound β-arrestin provide an optical detection of GPCR signal transduction that is as sensitive as any chemical amplification normally produced by second messenger cascades. Therefore, βarr2-GFP is not only exquisitely adept as a biosensor for monitoring GPCR activation, but represents an excellent tool to study the kinetics and specificity of components involved in the regulation of GPCR activity. Furthermore, when used as an optical sensor, βarr2-GFP provides the unique potential to unite orphan GPCRs with their corresponding ligands.

REFERENCES


Fig. 6. Agonist-mediated βarr2-GFP translocation to the dopamine D₁ receptor in HEK-293 cells. A–D, the dopamine D₁ receptor and βarr2-GFP were expressed in HEK-293 cells in the absence and presence of GRK2 as described under “Experimental Procedures.” Paired images of cells (A and B, C and D) are shown before and after agonist treatment. Prior to agonist treatment (A, C) βarr2-GFP is found uniformly throughout the cytosol. Upon addition of 20 µm dopamine βarr2-GFP can be observed to translocate to the plasma membrane (B, D). The relative amount of βarr2-GFP translocated to the plasma membrane is enhanced by the presence of GRK2 (agonist-treated, GRK-untreated cell group B compared with agonist-treated, GRK2-treated cell group D). Bar = 10 µm.