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 this problem using a β-arrestin2/green fluorescent protein conjugate (βarr2-GFP). It provides a real-time and single cell based assay to monitor GPCR activation and GPCR-G protein-coupled receptor kinase or 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.

EXPERIMENTAL PROCEDURES

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1The abbreviations used are: GPCR, G protein-coupled receptor; GFP, green fluorescent protein; βarr2-GFP, β-arrestin2 green fluorescent protein conjugate; GRK, G protein-coupled receptor kinase; β2AR, β2-adrenergic receptor.

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**RESULTS**

GFP fluorescence 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 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 β2ARs 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 2ARs, strongly suggesting that the primary targeted site of β-arrestin is the β2AR.

βarr2-GFP translocation to β2ARs is not limited to HEK-293
cells, but is also observable in COS-7 cells (Fig. 4). Consistent with their relatively larger surface area and lower efficiency of β2AR sequestration compared with HEK-293 cells, agonist-mediated βarr2-GFP translocation was less apparent in the COS cells (Fig. 4, A and corresponding image C) (16). However, by coexpressing GRK2, the agonist-mediated βarr2-GFP translocation could be enhanced (Fig. 4, B and corresponding image D), suggesting that GRK phosphorylation increases the affinity of the receptor for β-arrestin (22). To further characterize the role of GRK phosphorylation in β-arrestin translocation, we examined the ability of a GRK phosphorylation-impaired mutant Y326A-β2AR to support β-arrestin redistribution (11, 17, 23, 24). Consistent with its inability to be phosphorylated by endogenous GRKs, the Y326A-β2AR mutant did not induce βarr2-GFP translocation with agonist exposure (Fig. 5A). However, with overexpression of GRK2 and agonist treatment, the Y326A mutant-mediated βarr2-GFP translocation (Fig. 5B) was indistinguishable from β2AR-mediated translocation (7, 25–26). These results indicate βarr2-GFP translocation not only accurately monitors the biology of the GPCR activation process but the GPCR phosphorylation state as well.

To establish that agonist-induced βarr2-GFP translocation represents a general property of GPCR activation and is not limited to the β2AR, other members of the GPCR superfamily were evaluated for their ability to mediate the movement of βarr2-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 angiotensin, α- and β-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 βarr2-GFP. Moreover, the increase in βarr2-GFP translocation was enhanced in the presence of overexpressed GRK2 (Fig. 6D).

**DISCUSSION**

In this work we demonstrate that β-arrestin interacts with GPCRs immediately following agonist stimulation and GRK phosphorylation. βarr2-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 βarr2-GFP, with the magnitudes of plasma membrane fluorescence signals ranging up to 10–20-fold above the intracellular background. While β-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-β2AR mutant, the mutant-mediated βarr2-GFP translocation is indistinguishable from the wild type β2AR-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. 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 unlabelled 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.

FIG. 6. Agonist-mediated βarr2-GFP translocation to the dopamine DA1 receptor in HEK-293 cells. A–D, the dopamine DA1 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 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.

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