Distinct conformations of GPCR–β-arrestin complexes mediate desensitization, signaling, and endocytosis

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β-Arenergic receptors (βARs) interact with G protein-coupled receptors (GPCRs) to desensitize G protein signaling, to initiate signaling on their own, and to mediate receptor endocytosis. Prior structural studies have revealed two unique conformations of GPCR–βarr complexes: the “tail” conformation, with βarr primarily coupled to the phosphorylated GPCR C-terminal tail, and the “core” conformation, where, in addition to the phosphorylated C-terminal tail, βarr is further engaged with the receptor transmembrane core. However, the relationship of these distinct conformations to the various functions of βarr is unknown. Here, we created a mutant form of βarr lacking the “finger-loop” region, which is unable to form the core conformation but retains the ability to form the tail conformation. We find that the tail conformation preserves the ability to mediate receptor internalization and βarr signaling but not desensitization of G protein signaling. Thus, the two GPCR–βarr conformations carry out distinct functions.

GPCR | arrestin | endocytosis | signaling | desensitization

Over the past decade, significant efforts have been made to understand the molecular properties and regulatory mechanisms that control the function of β-arrestin (βarr) interactions with G protein-coupled receptors (GPCRs) (1, 2). Once activated, GPCRs initiate a highly conserved signaling and regulatory cascade marked by interactions with: (i) heterotrimeric G proteins, which mediate their actions largely by promoting second-messenger generation (3); (ii) GPCR kinases (GRKs), which phosphorylate activated conformations of receptors (4); and (iii) βarrs, which bind to the phosphorylated receptors to mediate desensitization of G protein signaling and receptor internalization (5, 6). In addition, to their canonical function of desensitization and internalization, βarrs have been appreciated as independent signaling units by virtue of their crucial role as both adaptors and scaffolds for an increasing number of signaling pathways (7–11).

There are two driving forces that mediate βarr interactions with an activated GPCR: phosphorylation of the C-terminal tail of the receptor by GRKs and/or binding to the transmembrane core of the receptor. How each of these interactions contributes to βarr functionality remains unclear. Moreover, GPCRs tend to either interact with βarr transiently, termed “class A” GPCRs [e.g., β2-adrenergic receptor (β2AR)], or tightly, known as “class B” GPCRs [e.g., vasopressin type 2 receptor (V1R)]. For the current study, we use a previously described chimeric β2/V1R construct, which comprises the β2AR with its C-terminal tail exchanged with the V1R C-terminal tail (12–14). The β2/V1R construct provides an ideal system for studying a GPCR–βarr complex in vitro, because it maintains identical pharmacological properties to the WT β2AR and has a robustly increased class B affinity for βarr1, which allows stable β2/V1R–βarr complexes to be formed and purified.

Structural insights have shed some light onto the complexity of the interaction between GPCRs and βarrs. A recent structural study of a constitutively active rhodopsin–arrestin fusion protein revealed high-resolution information about a single conformation of the complex in which the arrestin engaged via the transmembrane core of the receptor (12). However, negative-stain electron microscopy (EM) analysis of an antigen-binding fragment 30 (Fab30)–stabilized β2/V1R–βarr1–Fab30 complex demonstrated that the β2/V1R–βarr1 complex assumes two unique conformations: one in which ~63% of the βarr1 in the complex is bound only to the phosphorylated receptor C-terminal tail and appears to hang from the receptor (“tail” conformation) and a second more fully engaged conformation representing ~37%, in which, in addition to the tail interaction, the

Significance
β-Arenergic receptors (βARs) interact with G protein-coupled receptors (GPCRs) to desensitize G protein signaling, initiate signaling on their own, and mediate receptor endocytosis. Using a panel of GPCRs believed to couple differently to βarrs, we demonstrate how distinct conformations of GPCR–βarr complexes are specialized to perform different subsets of these cellular functions. Our results thus provide a new signaling paradigm for the understanding of GPCRs, whereby a specific GPCR–βarr conformation mediates receptor desensitization, and another drives internalization and some forms of signaling.

Acknowledgments and disclosures

Reviewers: A.T., University of Glasgow; and J.T., University of California, San Diego. Conflict of interest statement: R.J.L. is a cofounder and shareholder of Trevena. Freely available online through the PNAS open access option.

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www.pnas.org/cgi/doi/10.1073/pnas.1701529114

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finger-loop region (FLR) of βarr1 inserts into the transmembrane core of the receptor (“core” conformation) (13).

It is not known whether different GPCR–βarr conformations mediate distinct functional outputs. Thus, we sought to identify βarr1 mutants that predominantly form complexes with β2V2R in one or the other conformation, and then to test their ability to promote βarr-mediated internalization, signaling, and desensitization of G protein signaling.

**Results**

We focused our mutagenesis approach on the FLR of βarr1 because this region mediates an essential interaction with the receptor transmembrane core (13, 15) that stabilizes the GPCR–βarr complex core conformation (16). Disrupting this interaction through βarr1 mutagenesis, we reasoned, would allow us to obtain a βarr1 that predominantly forms GPCR–βarr tail conformation complexes, and not any core-conformation complexes, when bound to GPCRs.

To identify βarr1 mutants that primarily form β2V2R–βarr1 complexes in the tail conformation, we devised a method to form (and purify) these complexes on a small scale (Fig. 1A), and then applied single-particle classification analysis using negative-stain EM to assess their structural features (Fig. 1B–E). Furthermore, we developed a camelid nanobody, Nb32, which binds to and stabilizes active βarr1 that predominantly complexes with β2V2R in the core conformation (Fig. 1B–F and Figs. S1 and S2). Using our method, the addition of Nb32 to the β2V2R–βarr1–Fab30 complex increased the percentage of β2V2R–βarr1 complexes in the core conformation from 34 to 63% (Fig. 1F and Figs. S1 and S2), thus allowing a more precise assessment of βarr1 mutants defective in their ability to form β2V2R–βarr1 core-conformation complexes.

None of the mutations in the FLR of βarr1 that were tested prevented βarr1 from forming complexes with β2V2R as analyzed by pull-down assays and EM (Fig. 1 and Figs. S1 and S2). However, several mutants severely reduced the ability of βarr1 to bind to receptor via the core conformation in the β2V2R–βarr1–Fab30 complexes.

**Fig. 1.** T4 lysozyme (T4L)β2V2R–βarr1 complexes formed and analyzed via EM with a newly developed functional purification method. (A) Schematic representation of the purification method to generate (T4L)β2V2R–βarr1 complexes. (B) Coomassie gel showing WT βarr1 interaction with (T4L)β2V2R in the absence or presence of conformation-stabilizing antibodies (Fab30, Nb32). IP, immunoprecipitation. (C) Representative negative-stain raw EM image of (T4L)β2V2R–βarr1–Fab30 complexes. (D) Class averages of the (T4L)β2V2R–βarr1–Fab30 complexes (Top) and (T4L)β2V2R–βarr1–Fab30–Nb32 complexes (Bottom) from negative-stain EM classification analysis. (E) Representative class averages (with cartoon representations) of the (T4L)β2V2R–βarr1–Fab30 complex in the tail and core conformations. (Scale bars: C, 20 nm; D and E, 10 nm.) (F) Summarized results of the different βarr1 FLR constructs tested for their ability to form the (T4L)β2V2R–βarr1–Fab30 core conformation in the presence or absence of Nb32. Note that the tail conformation encompasses all those (T4L)β2V2R–βarr1 complexes that are not in the core conformation.
complex, even in the presence of Nb32 (Fig. 1F). Most notable is the βarr1 (ΔFLR) mutant (Fig. 1F, construct 2), with the entire FLR removed, which led to a substantial decrease in the core conformation of the β2V3R–βarr1–Fab30 complex even in the presence of Nb32. Together, these results demonstrate that the βarr1 (ΔFLR) mutant is strongly impaired in its ability to interact with the receptor transmembrane core, and thus serves as a model for βarr1 that forms a complex with the β2V3R predominantly in the tail conformation.

Next, using the β2V3R, the cellular functionality of βarr1 (ΔFLR) was confirmed using well-established βarr1 recruitment and internalization assays (Fig. S3A). Removal of the FLR did not impair agonist-mediated recruitment of βarr1 or βarr1-mediated receptor internalization, indicating that βarr1 (ΔFLR) can perform these functions for the β2V3R (Fig. S3A). We then set out to test whether distinct conformations of GPCR–βarr1 complexes determine differential functional outcomes by using an array of well-established biochemical, cellular, and biophysical assays. In addition to the chimeric β2V3R, its more physiological relatives, β2AR and V3R, were studied in parallel.

Classical GPCR activation promotes translocation of βarr1 from the cytosol to the GPCRs in the plasma membrane, and subsequently facilitates intracellular trafficking of GPCRs to endosomes (14). Thus, to ascertain the impact of the βarr1 (ΔFLR) mutant on recruitment to the β2AR, β2V3R, and V3R, as well as subsequent trafficking, confocal microscopy imaging was applied. Using this approach, we tracked the cellular localization of N-terminal SNAP-tagged GPCRs (SNAP-β2AR, SNAP-β2V3R, or SNAP-V3R) prelabeled with SNAP-Surface 649 fluorescent substrate and GFP-βarr1 (WT) or GFP-βarr1 (ΔFLR) in βarr1/βarr2 double-knockout (DKO) HEK293 cells following agonist treatment (16). The experiments demonstrate that βarr1 (WT or ΔFLR) is recruited to both the β2V3R and V3R, and that both mediate receptor internalization to endosomes, 30 min post-stimulation, to a similar extent (Fig. 2). In contrast, only the βarr1 (WT), but not the βarr1 (ΔFLR), is recruited to the β2AR upon agonist stimulation followed by receptor internalization.

The cellular trafficking pattern of βarr1 (WT or ΔFLR) was further quantified using bioluminescence resonance energy transfer (BRET) biosensors to monitor recruitment to the plasma membrane [Renilla reniformis green fluorescent protein (rGFP)-CAAX as a plasma membrane marker] and early endosome (rGFP-FYVE as an early endosomal marker) upon agonist stimulation of the three GPCRs in DKO HEK293 cells (17) (Fig. S3A). Agonist stimulation of β2AR, β2V3R, or V3R caused an increase in the BRET signal between RlucII-βarr1 (WT) and the plasma-membrane rGFP-CAAX biosensor (Fig. 3B and Fig. S3B). With the βarr1 (ΔFLR), agonist stimulation of either β2V3R or V3R also increased the BRET signal between RlucII-βarr1 (ΔFLR) and rGFP-CAAX, but to a slightly reduced extent for the β2V3R compared with RlucII-βarr1 (WT) (Fig. 3B and Fig. S3B). These findings indicate that both β2V3R and V3R are not dependent, to any large extent, on the core interaction to form a stable complex with βarr1. However, for the β2AR, there was no increased BRET signal between RlucII-βarr1.

![Fig. 2. Cellular localization of SNAP-β2AR (A), SNAP-β2V3R (B), or SNAP-V3R (C), prelabeled with SNAP-surface 649 fluorescent substrate (red) and GFP-βarr1 (WT or ΔFLR) (green), visualized by confocal microscopy. Cellular localization of fluorescently tagged proteins is shown before agonist addition (0 min) or 30 min after agonist stimulation. To stimulate the GPCRs, 1 μM BI-167107 was applied for the SNAP-β2AR and SNAP-β2V3R, and 100 nM arginine vasopressin was applied for the SNAP-V3R (100× objective, n = 3 independent experiments, n = 20–50 cells per experiment). (Scale bar: 10 μm.)](image-url)
(ΔFLR) and rGFP-CAAX upon agonist stimulation, suggesting that the βarr1 (ΔFLR) is unable to be recruited to this GPCR (Fig. 3B and Fig. S3B).

A significant, but slightly reduced, agonist-promoted BRET increase between Rlucl-βarr1 (ΔFLR) and the early endosomal marker, rGFP-FYVE, biosensor was detected compared with βarr1 (WT) for the β2V2R or V2R. These results suggest that βarr1 (ΔFLR) is capable of mediating internalization of the β2V2R or V2R to early endosomes, although to a lesser extent than βarr1 (WT) (Fig. 3C and Fig. S3B). In agreement with previous work (16) on the β2AR and its interaction with βarr1 showing that this class A GPCR recycles quickly and that βarr1 is not present in endosomes, no change in the BRET signal was detected between Rlucl-βarr1 (WT or ΔFLR) and rGFP-FYVE following agonist treatment of β2AR-transfected DKO HEK293 cells (Fig. 3C and Fig. S3B).

The scaffolding function of βarrs, as signal transducers, has been characterized for multiple signaling proteins, including c-Src (18, 19). Formation of GPCR-βarr1-c-Src ternary complexes has been demonstrated to regulate multiple cellular functions downstream of various GPCRs (20). Thus, to investigate the capacity of βarr1 in the GPCR–βarr1 tail conformation to scaffold c-Src, we evaluated the ability of βarr1 (WT or ΔFLR) to interact with c-Src upon activation of β2AR, β2V2R, or...
V₂R in DKO HEK293 cells by communoprecipitation. As expected, βarr1 (WT) effectively binds c-Src upon stimulation of all three GPCRs (Fig. 4A and B). We also observed that the ability of the βarr1 (ΔFLR) to scaffold c-Src, upon stimulation of the β₂V₂R and V₂R, was slightly reduced relative to βarr1 (WT) (Fig. 4A and B). In contrast, βarr1 (ΔFLR) does not interact with c-Src upon β₂AR stimulation, as might be expected, because βarr1 (ΔFLR) is not recruited to β₂AR. The scaffolding function of βarr1 (ΔFLR) was further explored by Glutathione Sepharose (GST) pull-down assays using purified 6xHis-βarr1 (WT or ΔFLR) and GST–c-Src either in the absence or presence of the phosphorylated V₂R C-terminal peptide (V₂Rpp). In the presence of V₂Rpp, an increased interaction was observed between βarr1 (WT or ΔFLR) and GST–c-Src (Fig. S3C). The βarr1 (ΔFLR) mutant is slightly impaired relative to βarr1 (WT) with respect to scaffolding c-Src in vitro, a trend also observed in our aforementioned cellular studies of both βarr1–c-Src scaffolding and βarr1-mediated GPCR internalization to endosomes (Figs. 3C and 4A).

βarr1 is known to promote desensitization of GPCR-stimulated G protein-mediated signaling. The mechanism underlying βarr1-mediated desensitization is thought to involve the interaction between βarr and the receptor core; this core conformation, presumably, sterically blocks the G protein-binding site in the receptor core (21). To assess the importance of the FLR of βarr1 for receptor desensitization directly, we monitored the attenuation of agonist-stimulated heterotrimeric Gs protein signaling, measured here as cAMP accumulation, in either the DKO (for the β₂AR) or a βarr1/βarr2/β₂AR triple-knockout (for the β₂V₂R and V₂R) HEK293 cell line expressing ICUE2, a fluorescence resonance energy transfer biosensor-detecting cytoplasmic cAMP (22). This ICUE2 biosensor measures cAMP concentration in real time, and thus represents equilibrium between production and degradation of cAMP. β₂AR, β₂V₂R, and V₂R were all expressed at near-endogenous levels (~100–400 fmol/mg), together with GRK2-CAAX, to ensure effective receptor phosphorylation and βarr1 recruitment upon agonist challenge. For all three GPCRs, agonist stimulation led to a rapid onset of cAMP generation, and this signal was only minimally reduced throughout the 30-min duration of the experiment (Fig. 4C).

We next coexpressed βarr1 (WT or ΔFLR) to test its ability to desensitize G protein signaling. Within the first 2 min of agonist challenge, β₂AR, β₂V₂R, and V₂R all stimulated cAMP production to a similar extent. Beyond 2 min, βarr1 (WT) attenuated the cAMP responses differently among these receptors (Fig. 4C), and most prominently for the WT β₂AR, where the addition of βarr1 (WT) led to rapid, but incomplete, desensitization. In contrast, βarr1 (ΔFLR) did not mediate any desensitization of the β₂AR-stimulated cAMP response because it is not recruited

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**Fig. 4.** Functional outcomes of different GPCR-βarr1 complex conformations. (A) Schematic representation of the functional outcomes mediated by GPCR–βarr1 complex tail conformation and GPCR–βarr1 complex core conformation. (B) βarr1-mediated scaffolding of c-Src upon activation of β₂AR, β₂V₂R, or V₂R. HEK293 DKO cells were transfected with plasmids for β₂AR, β₂V₂R, or V₂R, c-Src, and HA-βarr1 (WT or ΔFLR). Serum-starved cells were stimulated with or without agonist Bi-167107 (1 μM) or AVP (100 nM) for 10 min and then cross-linked using dithiobis(succinimidyl propionate); finally, anti-HA beads were used to pull down βarr1 (WT or ΔFLR). The amount of total c-Src bound to HA-βarr1 (WT or ΔFLR) was determined by immunoblotting (IB). Data represent the mean ± SE of four to five experiments. One-way ANOVA was performed to determine statistical differences between basal and agonist-stimulated states (****P < 0.0001), or agonist-stimulated states in βarr1 (WT)– and βarr1 (ΔFLR)-transfected cells (###P < 0.001, ####P < 0.0001). (C) βarr1-mediated desensitization of Gs-promoted cAMP generation by the β₂AR, β₂V₂R, or V₂R. Real-time cAMP measurements, using ICUE2-expressing HEK293 cells, in response to agonist stimulation of β₂AR, β₂V₂R, or V₂R are shown. For the β₂AR and β₂V₂R, 1 μM Bi-167107 was used to stimulate cells. For V₂R, 100 nM AVP was used to stimulate cells. For each GPCR, control plasmid (Mock, black), βarr1 (WT) (blue), or βarr1 (ΔFLR) (red) was transfected. Surface expression of each GPCR was matched within each βarr1 transfection condition. Data represent the mean ± SE of three to four experiments and n ≥ 44 cells. Area under the curve (A.U.C.) from 2 min after agonist stimulation to the end of the experiment was used to calculate desensitization of the cAMP response for each GPCR, and one-way ANOVA was performed to determine statistical differences relative to Mock (**P < 0.01, ***P < 0.001) and βarr1 (WT) (P < 0.05, ###P < 0.001) responses. Forsk, forskolin.

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to this receptor. βarr1 (WT)-mediated desensitization was also observed at the β2V3R-stimulated cAMP response (Fig. 4C). βarr1 (WT) did not have a significant effect on V3R-stimulated cAMP signaling, which agrees with previous work (23). Most strikingly, expression of βarr1 (ΔFLR) did not lead to any significant desensitization of G protein signaling for any of the GPCRs tested (Fig. 4C). These results (Fig. 4A and C) demonstrate that the FLR domain of βarr1, presumably through its role in forming the core interaction, is crucial for βarr1-mediated desensitization of G protein signaling.

Discussion

Our results can be interpreted in the context of the classification of GPCRs according to the strength of their interaction with βarRs. Class A GPCRs, such as the β2AR, bind βarRs relatively weakly and dissociate from them in the course of internalization. They thus recycle rapidly to the plasma membrane. Class B GPCRs, such as the V3R or the β1V2R chimera, bind βarRs much more tightly and, once internalized, remain bound to βarRs and resident in endosomes for significant periods of time. They recycle only slowly to the plasma membrane. For class B GPCRs, the GPCR–βarr complex, in the tail conformation, appears to be capable of promoting βarr-mediated receptor internalization and some forms of signaling, but not desensitization of G protein signaling, which appears to be the exclusive purview of the core-conformation complex (Fig. 4A). A recent study showed that some βarr-mediated functions are maintained when recruited to a potential core-deficient GPCR mutant, which supports our conclusions with respect to the function of the tail conformation complex (24). However, the study did not experimentally demonstrate any biological role of the core conformation. Our finding that the core-conformation complex appears to be crucial for mediating desensitization is in agreement with the classical notion that G proteins and βarRs compete for overlapping binding sites in the receptor transmembrane core (21).

Interestingly, for the class A β2AR, which binds βarRs more weakly, the tail conformation complex appears to be too unstable to lead to effective recruitment of the βarr1 (ΔFLR). Our data thus suggest that for such GPCRs, the tail conformation complex might not exist in a stable enough form to participate in βarr-mediated activities.

In addition, we have recently demonstrated that some GPCRs, such as the β2V3R and V3R but not the β1V2R, can form GPCR–Gs–βarr “megaplexes,” and thus activate G protein from internalized compartments (16). In these megaplexes, the receptor binds βarr in the tail conformation complex. Interestingly, in the current study, we find a clear correlation between the GPCRs that form GPCR–βarr1 tail conformation complexes and GPCRs that can activate G protein from internalized compartments. In contrast, GPCRs that rely more heavily on the core conformation do not seem to activate G protein after being internalized by βarr.

Acknowledgments

We thank L. Barak for generous gifts of plasmids encoding GFP-βarr1. We thank C.-R. Liang, L.-L. Gu, and J.-M. Shan for synthesizing Bi-16/1707 compound. We thank C. Cahill, P. Achacoso, S. Johnson, C. Le Gouill, A. Laperrière, M. Walters, M. Delong, M. Plue, T. Milledge, D. Capel, and X. Jiang for support and discussion. This work received support from NIH Grants F30HL129803 (to T.J.C.), T32HL07101 (to A.W.K.), DK900165 (to G.S.), and HL16037 (to R.J.L.); the Danish Council for Independent Research & Lundbeck Foundation (A.R.B.T.); a Canadian Institutes of Health Research (CIHR) postdoctoral fellowship (to B.P.); and CIHR Grant MOP10501 (to M.B.); JST, PRESTO (to A.I.), and AMED-CREST (to J.A.); and a Howard Hughes Medical Institute (HHMI) Medical Research Fellowship to A.H.N. R.J.L. is an HHMI Investigator and a cofounder and shareholder of Trevnea. M.B. holds a Canada Research Chair in Signal Transduction and Molecular Pharmacology.