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

Thomas J. Cahill IIIa,b,1, Alex R. B. Thomsenb,1, Jeffrey T. Tarraschc, Bianca Plouffea, Anthony H. Nguyena,f, Fan Yanga, Li-Yin Huangg, Alem W. Khahash, Daniel L. Bassoni, Bryant J. Gavino, Jane E. Lamedina, Sarah Triestid,j, Arun K. Shuklaa,2, Benjamin Bergerb, John Little IVb, Albert Antarb, Adi Blancb, Chang-Xiu Qug, Xin Chenk, and Robert J. Lefkowitzab,3

Arun K. Shuklaa,2, Benjamin Bergerb, John Little IVb, Albert Antarb, Adi Blancb, Chang-Xiu Qug, Xin Chenk, serves the ability to mediate receptor internalization and to form the tail conformation. We find that the tail conformation pre-receptor transmembrane core. However, the relationship of these dis-

3To whom correspondence should be addressed. Email: lefko001@receptor-biol.duke.edu.

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) 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 βarrs is unknown. Here, we created a mutant form of βarr lacking the “loop-ring” 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 can carry out distinct functions.

Significance

β-Arestrins (βarrs) 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 desensitization, and another drives internalization and some forms of signaling.


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1T.J.C. and A.R.B.T. contributed equally to this work.

2Present address: Department of Biological Sciences and Bioengineering, Indian Institute of Technology, Kanpur 208016, India.

3To whom correspondence should be addressed. Email: lefko001@receptor-biol.duke.edu.

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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 complex formation. Table 1 summarizes the results of the different βarr1 FLR constructs tested for their ability to form β2V2R–βarr1 complexes in the presence or absence of Nb32. Note that the tail conformation encompasses all those 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 β₂V₂R–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 β₂V₂R predominantly in the tail conformation.

Next, using the β₂V₂-R, 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 β₂V₂ (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 β₂V₂R, its more physiological relatives, β₂AR and V₂R, 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 βarr1 (ΔFLR) mutant on recruitment to the β₂AR, β₂V₂R, and V₂R, 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-β₂V₂R, or SNAP-V₂R) prelabeled with SNAP-Surface 649 fluorescence 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 β₂V₂R and V₂R, 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. 3A). Agonist stimulation of β₂AR, β₂V₂R, or V₂R 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 β₂V₂R or V₂R also increased the BRET signal between RlucII-βarr1 (ΔFLR) and rGFP-CAAX, but to a slightly reduced extent for the β₂V₂R compared with RlucII-βarr1 (WT) (Fig. 3B and Fig. S3B). These findings indicate that both β₂V₂R and V₂R 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](image_url)
β expressed as net BRET absolute values, represent the mean (±SE, are pooled from four to six experiments, and are analyzed using either a paired t test (two conditions) or one-way ANOVA with Tukey’s multiple comparisons post hoc test (three or more conditions).

A significant, but slightly reduced, agonist-promoted BRET increase between RlucII-βarr1 (ΔFLR) and the early endosomal marker, rGFP-CAAX, 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 RlucII-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

Fig. 3. Interaction between βarr1 (WT or ΔFLR) and either rGFP-CAAX (plasma membrane marker) or rGFP-FYVE (early endosomal marker) upon agonist stimulation of β2AR, β2V2R, or V2R. (A) Schematic representation of the experimental design used to monitor agonist-promoted BRET between RlucII-βarr1 (WT or ΔFLR) and rGFP-CAAX or rGFP-FYVE. (B) BRET concentration–response experiments assessing the agonist-stimulated RlucII-βarr1 (WT or ΔFLR) recruitment to plasma membrane-located rGFP-CAAX. Upon agonist addition, a difference in BRET was detected between βarr1 (WT) and β2AR (P = 0.0022), but not between βarr1 (ΔFLR) and β2AR (P = 0.4306). Agonist-mediated changes in net BRET between βarr1 (WT) and βarr1 (ΔFLR) were detected for both the β2AR (P = 0.0015) and β2V2R (P < 0.0001), but not for V2R (P = 0.0820). (C) BRET concentration–response experiments assessing the agonist-stimulated RlucII-βarr1 (WT or ΔFLR) localization to early endosomal-located rGFP-FYVE. Upon agonist addition, no BRET difference was detected between either βarr1 (WT) or βarr1 (ΔFLR) and β2AR (P = 0.4188 or P = 0.9016, respectively). Agonist-mediated changes in net BRET between βarr1 (WT) and βarr1 (ΔFLR) were detected for β2V2R (P = 0.0034) and V2R (P = 0.0014), but not for β2AR (P = 0.9057). In all experiments, BRET was measured 30 min following addition of agonist or vehicle. To stimulate the GPCRs, 1 μM Bi-167107 was applied for the β2AR and β2V2R, and 100 nM arginine vasopressin (AVP) was applied for the V2R. Data are expressed as net BRET absolute values, represent the mean ± SE, are pooled from four to six experiments, and are analyzed using either a paired t test (two conditions) or one-way ANOVA with Tukey’s multiple comparisons post hoc test (three or more conditions).
V2R in DKO HEK293 cells by coimmunoprecipitation. As expected, βarr1 (WT) effectively binds c-Src upon stimulation of all three GPCRs (Fig. 4 A and B). We also observed that the ability of the βarr1 (∆FLR) to scaffold c-Src, upon stimulation of the β2V2R and V2R, was slightly reduced relative to βarr1 (WT) (Fig. 4 A and B). In contrast, βarr1 (ΔFLR) does not interact with c-Src upon β2AR stimulation, as might be expected, because βarr1 (∆FLR) 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. 3C). The βarr1 (∆FLR) 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...
to this receptor, βarr1 (WT)-mediated desensitization was also observed at the β2V2R-stimulated cAMP response (Fig. 4C). βarr1 (WT) did not have a significant effect on V1R-stimulated cAMP signaling, which agrees with previous work (23). Most significantly, 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. 4 A 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 βarfs. Class A GPCRs, such as the β2AR, bind βarfs 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 V1R or the β2V2R chimera, bind βarfs much more tightly and, once internalized, remain bound to βarfs 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 formation, 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 βarfs compete for overlapping binding sites in the receptor transmembrane core (21). Interestingly, for the class A β2AR, which binds βarr 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 β2V2R and V1R but not the β2AR, can form GPCR-βrs-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.

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