



# Sortase ligation enables homogeneous GPCR phosphorylation to reveal diversity in $\beta$ -arrestin coupling

Dean P. Staus<sup>a,b,1</sup>, Laura M. Wingler<sup>a,b,1</sup>, Minjung Choi<sup>c</sup>, Biswaranjan Pani<sup>a</sup>, Aashish Manglik<sup>d,e</sup>, Andrew C. Kruse<sup>f</sup>, and Robert J. Lefkowitz<sup>a,b,c,2</sup>

<sup>a</sup>Department of Medicine, Duke University Medical Center, Durham, NC 27710; <sup>b</sup>Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710; <sup>c</sup>Department of Biochemistry, Duke University Medical Center, Durham, NC 27710; <sup>d</sup>Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94158; <sup>e</sup>Department of Anesthesia and Perioperative Care, University of California, San Francisco, CA 94158; and <sup>f</sup>Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

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The ability of G protein-coupled receptors (GPCRs) to initiate complex cascades of cellular signaling is governed by the sequential coupling of three main transducer proteins, G protein, GPCR kinase (GRK), and  $\beta$ -arrestin. Mounting evidence indicates these transducers all have distinct conformational preferences and binding modes. However, interrogating each transducer's mechanism of interaction with GPCRs has been complicated by the interplay of transducer-mediated signaling events. For example, GRK-mediated receptor phosphorylation recruits and induces conformational changes in  $\beta$ -arrestin, which facilitates coupling to the GPCR transmembrane core. Here we compare the allosteric interactions of G proteins and  $\beta$ -arrestins with GPCRs' transmembrane cores by using the enzyme sortase to ligate a synthetic phosphorylated peptide onto the carboxyl terminus of three different receptors. Phosphopeptide ligation onto the  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR) allows stabilization of a high-affinity receptor active state by  $\beta$ -arrestin1, permitting us to define elements in the  $\beta_2$ AR and  $\beta$ -arrestin1 that contribute to the receptor transmembrane core interaction. Interestingly, ligation of the identical phosphopeptide onto the  $\beta_2$ AR, the muscarinic acetylcholine receptor 2 and the  $\mu$ -opioid receptor reveals that the ability of  $\beta$ -arrestin1 to enhance agonist binding relative to G protein differs substantially among receptors. Furthermore, strong allosteric coupling of  $\beta$ -arrestin1 correlates with its ability to attenuate, or "desensitize," G protein activation *in vitro*. Sortase ligation thus provides a versatile method to introduce complex, defined phosphorylation patterns into GPCRs, and analogous strategies could be applied to other classes of posttranslationally modified proteins. These homogeneously phosphorylated GPCRs provide an innovative means to systematically study receptor–transducer interactions.

G protein-coupled receptor |  $\beta$ -arrestin | sortase | allostery | phosphorylation

**G** protein-coupled receptors (GPCRs), a large family of plasma membrane receptors coupled to guanine nucleotide regulatory proteins, represent one of the most important mechanisms for transducing extracellular signals into specific cellular responses. Their important role in regulating many physiological processes makes them a common therapeutic target. Despite their ability to recognize a vast array of ligands (1), GPCRs have a highly conserved mechanism of action. Ligand binding to the extracellular orthosteric pocket induces conformational changes within the receptor transmembrane (TM) region (2), leading to the sequential intracellular coupling of three main transducer proteins: G protein, GPCR kinase (GRK), and  $\beta$ -arrestin ( $\beta$ arr) (3). More specifically, GPCR-dependent activation of the heterotrimeric G protein leads to the dissociation of the  $\alpha$ -subunit from the  $\beta\gamma$ -subunits, resulting in modulation of second messenger systems, such as cAMP (4). Subsequent GRK phosphorylation of specific serine/threonine residues within the receptor third intracellular loop (ICL3) or carboxyl (C)-terminal tail recruits  $\beta$ arr (5). The binding of  $\beta$ arr desensitizes

GPCR signaling by sterically blocking G protein coupling and promoting receptor internalization through interactions with AP2 and clathrin (6). Additionally,  $\beta$ arr can directly modulate cell signaling through G protein-independent pathways (7).

It is now well established that "biased" GPCR ligands can disproportionately regulate particular branches of receptor signaling, a phenomenon known as biased agonism (8). The selective activation of signaling pathways indicates that, although all three transducers specifically interact with agonist-bound GPCRs, their conformational specificities are not identical. However, the fundamental mechanisms underlying this differential coupling remain obscure, largely because events mediated by different transducers are intricately intertwined. In particular,  $\beta$ arr binds to receptors through a two-step process, initially interacting with GRK-phosphorylated residues and then coupling to the agonist-activated GPCR TM core (Fig. 1) (9). Biochemical and structural studies have demonstrated that binding to GPCRs' phosphorylated tails induces extensive conformational changes in  $\beta$ arr, including the extension of several loops implicated in  $\beta$ arr's interaction with GPCRs' TM bundle (10).

## Significance

**$\beta$ -Arrestin regulates G protein-coupled receptor (GPCR) signaling by interacting with two regions of agonist-activated receptors—the phosphorylated C terminus and the seven transmembrane helix bundle. The phosphorylation pattern on GPCRs is thought to be the primary driver of  $\beta$ -arrestin binding affinity and functional consequences. To more effectively delineate the relative contributions of these two interactions, we present an innovative strategy to homogeneously phosphorylate purified GPCRs—enzymatic ligation of a synthetic phosphopeptide. This approach unexpectedly revealed that different receptors with identical phosphorylation patterns exhibit dramatic variability in their ability to couple to  $\beta$ -arrestin through the transmembrane core. These differences could play an important role in tuning the balance of G protein- and  $\beta$ -arrestin-mediated cellular signaling pathways stimulated by each GPCR.**

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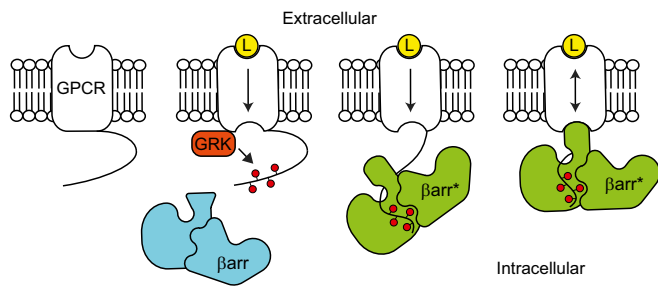
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<sup>1</sup>D.P.S. and L.M.W. contributed equally to this work.

<sup>2</sup>To whom correspondence should be addressed. Email: lefko001@receptor-biol.duke.edu.

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**Fig. 1.** Illustration showing the two-step binding mode of  $\beta$ -arrestin. Ligand (L) binding to the extracellular orthosteric binding pocket leads to conformational changes within the GPCR transmembrane region to influence intracellular transducer binding. The phosphorylation (red circles) of the receptor C terminus by GPCR kinase (GRK) initiates the recruitment of  $\beta$ -arrestin ( $\beta$ arr). Conformational changes induced in  $\beta$ arr ( $\beta$ arr\*) as a result of binding to the phosphorylated C terminus promotes coupling to the GPCR transmembrane core, which allosterically enhances ligand affinity.

Engagement of  $\beta$ arr with GPCRs' TM cores is believed to mediate particular functions of  $\beta$ arr, such as receptor desensitization, but efforts to understand the nature and consequences of this interaction have been hampered by its low affinity and its dependence on GRK phosphorylation. Obtaining uniformly phosphorylated receptors in a cellular context or *in vitro* has proven to be challenging. Here we present a method to generate homogeneously phosphorylated GPCRs by enzymatically ligating a synthetic phosphorylated peptide, removing the confounding variable of phosphorylation so that the effects of  $\beta$ arr's coupling to the TM cores can be isolated. This allows us to compare systematically how agonists allosterically influence the interactions of multiple transducers with multiple GPCRs, revealing unexpected diversity that may influence the balance of cellular signaling responses.

## Results

The binding of  $\beta$ arr to GPCRs is mainly initiated through an interaction with the phosphorylated receptor C terminus, and conformational changes induced in  $\beta$ arr by this interaction promote coupling to the receptor TM core (Fig. 1). Coimmunoprecipitation experiments confirm that heterotrimeric Gs protein, but not  $\beta$ arr1, can interact with purified nonphosphorylated  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR) (Fig. 2A).

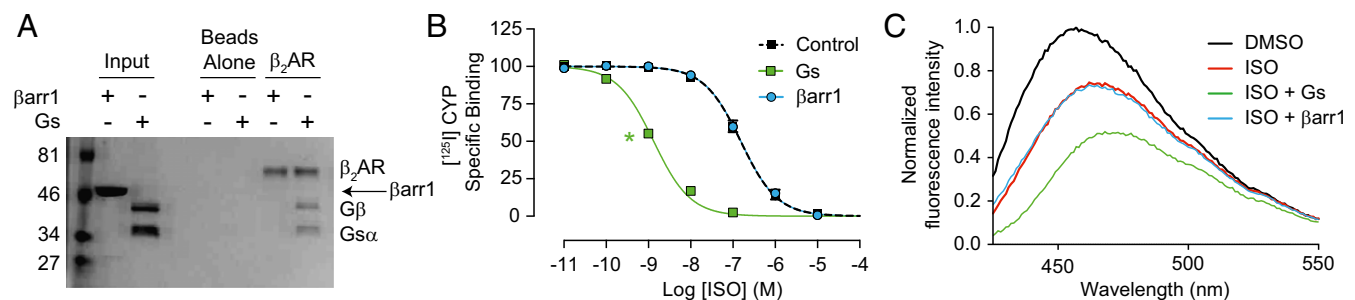
To verify that this apparent lack of interaction with  $\beta$ arr is not simply due to poor complex stability, we employed two assays capable of detecting complex formation *in situ*. First, we used competition radioligand binding to measure the allosteric effects of transducers on ligand binding to the receptor. As described by

the ternary complex model, first for G proteins and later for  $\beta$ arrs, ligand-induced changes in receptor conformation enhance the binding and affinity of transducers, which reciprocally increase ligand affinity by stabilizing an active receptor state (11, 12). When we reconstitute wild-type (WT)  $\beta_2$ AR in high-density lipoprotein (HDL) particles to mimic a cellular membrane environment (13), G protein enhances the affinity of the full agonist isoproterenol (ISO) for nonphosphorylated HDL- $\beta_2$ AR by nearly 1,000-fold, as expected, but  $\beta$ arr1 has no effect even at micromolar concentrations (Fig. 2B).

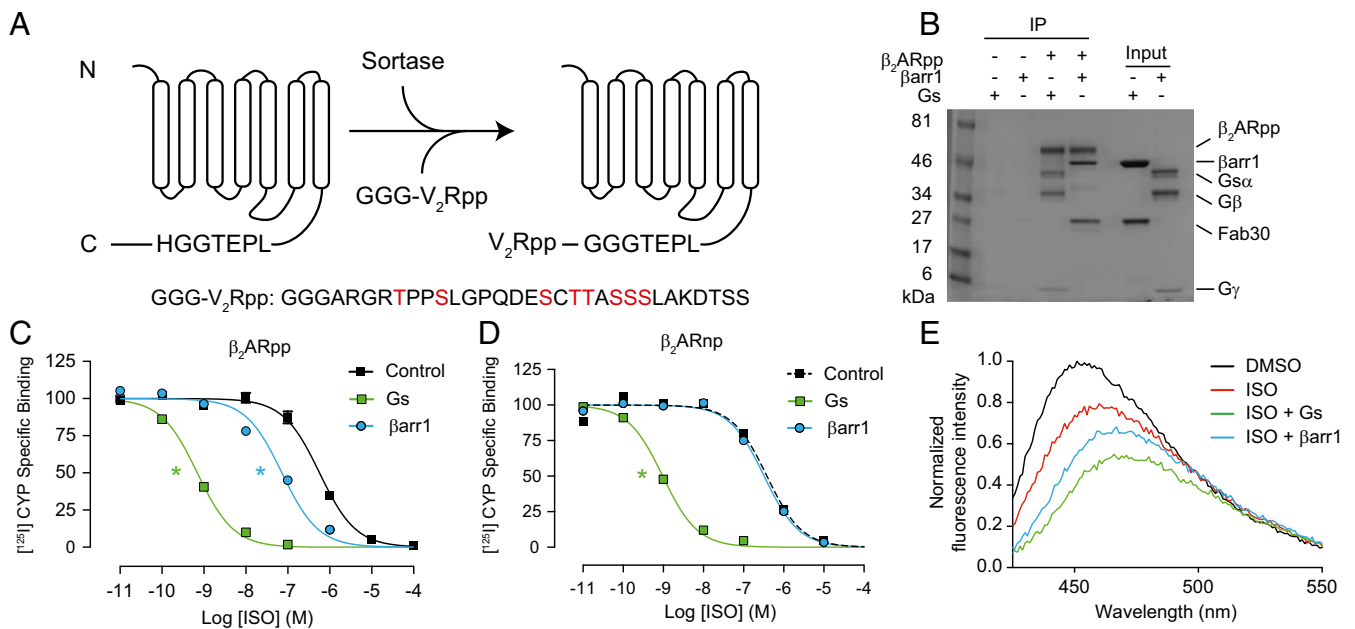
Second, to directly monitor  $\beta_2$ AR conformational changes associated with activation, we labeled C265 at the cytoplasmic end of TM6 with monobromobimane, an environmentally sensitive fluorophore. Receptor activation leads to an outward movement of TM6 that places the bimane label in a more solvent-exposed position, causing a decrease in fluorescence and a shift in  $\lambda_{\max}$  (14). Indeed, isoproterenol reduces  $\beta_2$ AR-bimane fluorescence compared with control (DMSO), and addition of Gs but not  $\beta$ arr1 further attenuates fluorescence (Fig. 2C). Taken together, these data clearly indicate that nonphosphorylated  $\beta_2$ AR fails to form a productive interaction with  $\beta$ arr.

We induced phosphorylation of the  $\beta_2$ AR by using the prokaryotic enzyme sortase to ligate a synthetic phosphorylated peptide onto the receptor C terminus (Fig. 3A and Fig. S1). This strategy quantitatively yields receptor with a defined, homogeneous phosphorylation pattern, which is difficult to achieve or validate with either *in cellulo* or *in vitro* GRK phosphorylation. We ligated a phosphopeptide (pp) derived from the C terminus of the vasopressin-2-receptor ( $V_2R$ ), given our previous crystallographic and biophysical data (10), which indicate that  $V_2R$ pp binds to  $\beta$ arr with high affinity and effectively primes it for interaction with GPCRs' TM core. In contrast to WT  $\beta_2$ AR (Fig. 2A), phosphorylated  $\beta_2$ AR ( $\beta_2$ ARpp) can immunoprecipitate both Gs and  $\beta$ arr1 (Fig. 3B).  $\beta$ Arr1 enhances isoproterenol affinity for the  $\beta_2$ ARpp by 9-fold, compared with 800-fold by Gs (Fig. 3C and Fig. S24). However, as for Gs,  $\beta$ arr1 does not increase the binding of the antagonist ICI-118,551 (Fig. S2B). The  $\beta$ arr1-mediated increase in agonist affinity requires phosphorylation of  $\beta_2$ ARpp, since ligation of a nonphosphorylated  $V_2R$  peptide or phosphatase treatment abrogates  $\beta$ arr1's allosteric effect (Fig. 3D and Fig. S2C). While  $\beta$ arr1 augments isoproterenol's decrease in the fluorescence of  $\beta_2$ ARpp-bimane, its effects are less profound than those of G protein (Fig. 3E), consistent with the  $\sim$ 100-fold difference in the cooperativity between G protein and  $\beta$ arr1 observed by radioligand binding (Fig. 3C). These findings suggest that despite binding to a similar pocket, G protein and  $\beta$ arr differ substantially in the strength of their allosteric interactions with the  $\beta_2$ AR TM core.

This defined system allows us to rigorously assess the contributions of specific regions within each protein that have been



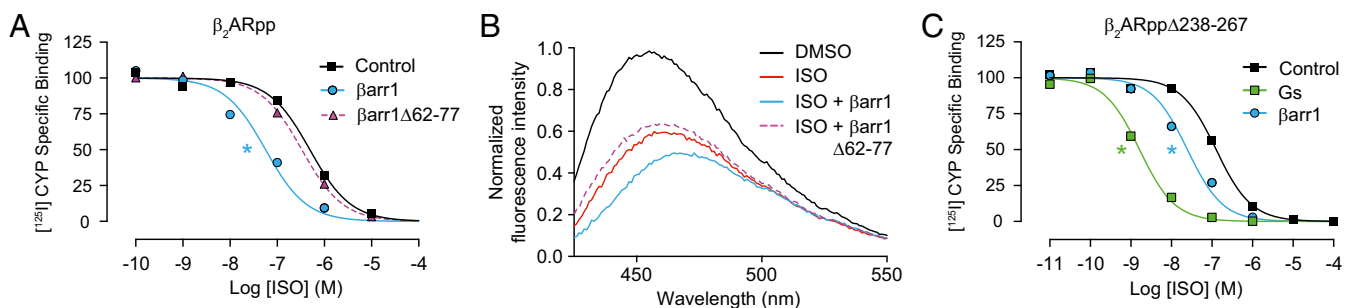
**Fig. 2.** Nonphosphorylated  $\beta_2$ AR interacts with Gs heterotrimer but not  $\beta$ -arrestin1. (A) Coomassie-stained gel showing the coimmunoprecipitation of Gs heterotrimer (Gs) or  $\beta$ -arrestin1 ( $\beta$ arr1) with isoproterenol (ISO)-bound FLAG- $\beta_2$ AR. Loading controls represent 10% of input. (B) Competition binding experiments using radiolabeled [ $^{125}$ I]-cyanopindolol (CYP). Gs increases ISO affinity for  $\beta_2$ AR HDLs (log  $IC_{50}$ :  $-8.88 \pm 0.03$ ) compared with no transducer (log  $IC_{50}$ :  $-6.82 \pm 0.03$ ), but  $\beta$ arr1 does not (log  $IC_{50}$ :  $-6.81 \pm 0.02$ ). Data shown are the mean of three independent experiments, with error bars representing SE. The green asterisk (\*) indicates a log  $IC_{50}$  value significantly different from the control curve ( $P < 0.05$ , one-way ANOVA). (C) The fluorescence emission spectrum of bimane-labeled  $\beta_2$ AR HDLs shows a rightward shift and decrease in fluorescence upon addition of ISO, indicative of receptor activation. The effects of ISO are enhanced by Gs but not  $\beta$ arr1. Data shown are representative of three independent experiments.



**Fig. 3.** Sortase ligation of a phosphopeptide onto the  $\beta_2$ AR restores its allosteric interaction with  $\beta$ -arrestin1. (A) Cartoon schematic of sortase ligation method. A synthetic phosphopeptide (pp) derived from the vasopressin-2-receptor ( $V_2R$ ) with three N-terminal glycine residues (GGG- $V_2Rpp$ ) is ligated onto receptors containing a C-terminal LPETGGH recognition motif. In the sequence of GGG- $V_2Rpp$  below the schematic, phosphorylated residues are highlighted in red. (B) Coomassie-stained gel showing the coimmunoprecipitation of heterotrimeric Gs and  $\beta$ -arrestin1 ( $\beta arr1$ ) with isoproterenol (ISO)-bound, phosphopeptide-ligated FLAG- $\beta_2$ AR ( $\beta_2ARpp$ ). Fab30 binds specifically to  $V_2Rpp$ -bound  $\beta arr1$  (10). Loading controls represent 10% of input. (C and D) Competition binding experiments using radiolabeled [ $^{125}$ I]-cyanopindolol (CYP) with HDLs containing (C)  $\beta_2ARpp$  or (D)  $\beta_2ARnp$  ligated to a nonphosphorylated version of the  $V_2R$  peptide ( $\beta_2ARnp$ ). Gs increases the affinity of ISO for both  $\beta_2ARpp$  and  $\beta_2ARnp$  HDLs (log  $IC_{50}$ :  $-9.15 \pm 0.03$ ,  $-9.02 \pm 0.04$ , respectively) compared with no transducer (log  $IC_{50}$ :  $-6.24 \pm 0.04$ ,  $-6.42 \pm 0.09$ , respectively), but  $\beta arr1$  only increases ISO affinity for  $\beta_2ARpp$  HDLs (log  $IC_{50}$ :  $-7.14 \pm 0.07$ ) and not  $\beta_2ARnp$  HDLs (log  $IC_{50}$ :  $-6.49 \pm 0.04$ ). Data shown in C and D are the mean of at least three independent experiments, with error bars representing SE, and asterisks (\*) indicate a log  $IC_{50}$  value significantly different from the control curve ( $P < 0.05$ , one-way ANOVA). (E) The effects of ISO on the HDL- $\beta_2ARpp$ -bimane fluorescence emission spectrum are enhanced by Gs and  $\beta arr1$ . Data shown are representative of three independent experiments.

implicated in mediating the TM core/ $\beta arr1$  interaction. For example, the “finger loop” region of  $\beta arr1$  is extended upon  $\beta arr1$ 's binding to phosphorylated receptors and is believed to insert into the TM core. We previously reported that this region was essential to observe an engaged conformation of  $\beta arr1$  with the TM core of in cellulose phosphorylated  $\beta_2AR$ , as assessed by negative stain electron microscopy using a  $\beta arr1$  finger loop-deleted mutant (15). This same mutant,  $\beta arr1\Delta 62-77$ , fails to stabilize an active state of  $\beta_2ARpp$  by competition radioligand binding (Fig. 4A) and bimane fluorescence (Fig. 4B), consistent with our previous findings.

On the receptor side, it has been suggested that ICL3 of the  $\beta_2AR$  is critical for engagement of  $\beta arr1$  with the TM core (16). The phosphopeptide-ligated version of a previously reported deletion mutant,  $\beta_2ARpp\Delta 238-267$ , retains a normal affinity for the agonist isoproterenol when reconstituted in HDL particles (Fig. 4C). Surprisingly, agonist affinity increases (~10-fold) in the presence of  $\beta arr1$  (Fig. 4C), quite comparable to  $\beta arr1$ 's effect on WT  $\beta_2ARpp$  (Fig. 3C). Together these data indicate that the finger loop of  $\beta arr1$ , but not the  $\beta_2AR$  ICL3, is required for the TM core interaction.



**Fig. 4.** The allosteric interaction between phosphorylated  $\beta_2AR$  and  $\beta$ -arrestin1 requires the finger loop of  $\beta$ -arrestin1 but does not require the third intracellular loop of the  $\beta_2AR$ . (A) In competition radioligand binding with  $\beta_2ARpp$  HDLs as described in Fig. 3C, a finger loop deletion mutant of  $\beta$ -arrestin1 ( $\beta arr1$ ) ( $\Delta 62-77$ ) has minimal effect on isoproterenol (ISO) binding (log  $IC_{50}$ : no transducer,  $-6.30 \pm 0.05$ ;  $\beta arr1$ ,  $-7.25 \pm 0.07$ ;  $\beta arr1\Delta 62-77$ ,  $-6.48 \pm 0.03$ ). (B)  $\beta arr1\Delta 62-77$  does not intensify the effects of ISO on the fluorescence spectrum of  $\beta_2ARpp$ -bimane HDLs. Data shown are representative of three independent experiments. (C) In competition radioligand binding with  $\beta_2ARpp$  HDLs containing a deletion of the third intracellular loop ( $\Delta 238-267$ ), both Gs (log  $IC_{50}$ :  $-8.85 \pm 0.03$ ) and  $\beta arr1$  (log  $IC_{50}$ :  $-7.63 \pm 0.06$ ) retain their ability to increase isoproterenol (ISO) affinity (no transducer, log  $IC_{50}$ :  $-6.91 \pm 0.04$ ). Data shown in A and C are the mean of at least three independent experiments, with error bars representing SE, and asterisks (\*) indicate a log  $IC_{50}$  value significantly different from the control curve ( $P < 0.05$ , one-way ANOVA).

For the  $\beta_2$ AR, the TM core's allosteric communication with G protein is substantially stronger than it is with  $\beta$ arr. To determine whether this is a conserved phenomenon among other GPCRs, we investigated the allosteric coupling of G protein and  $\beta$ arr at the muscarinic acetylcholine receptor 2 ( $M_2$ R) and  $\mu$ -opioid receptor (MOR). Using the sortase ligation strategy described above for the  $\beta_2$ AR, we ligated the  $V_2$ Rpp onto the C termini of purified  $M_2$ R ( $M_2$ Rpp) and MOR (MORpp) (Fig. S3A), reconstituted the receptors into HDL particles, and measured the allosteric coupling of their cognate G protein (Gi heterotrimer) and  $\beta$ arr using competition radioligand binding. We selected competitor ligands that are full agonists and have similar affinities for their respective receptors as isoproterenol does for the  $\beta_2$ AR. As observed for the  $\beta_2$ AR, G protein induces more than a 100-fold increase in agonist affinity for both  $M_2$ Rpp (carbachol, Fig. 5A) and MORpp (DAMGO, Fig. 5B), consistent with previous reports (17, 18).  $\beta$ Arr1 enhances agonist affinity for both  $M_2$ Rpp and MORpp in a phosphorylation-dependent manner (Fig. 5A and B and Fig. S3B and C), but interestingly,  $\beta$ arr1 increases carbachol affinity for  $M_2$ Rpp by 57-fold compared with only 2- and 9-fold for MORpp and  $\beta_2$ ARpp, respectively (Fig. 5A and B and Fig. S3D). A summary of transducer allosteric binding at each receptor is shown in Fig. 5C, where we observe a 100-fold difference between G protein and  $\beta$ arr effects on agonist affinity for the  $\beta_2$ ARpp and MORpp but less than a 3-fold difference with  $M_2$ Rpp. The comparable effects of G protein and  $\beta$ arr at the  $M_2$ Rpp are not carbachol specific but are also observed with the agonist iperoxo (Fig. S3E and F).  $\beta$ Arr1's effects at the  $M_2$ Rpp also appear to be dependent on the transmembrane core interaction, as deletion of the finger loop eliminates its allosteric coupling (Fig. S3G). Therefore, even for GPCRs which preferentially couple to the same G protein isoform, such as the  $M_2$ R and the MOR, allosteric communication with G protein does not always vary proportionally to allosteric communication with  $\beta$ arr.

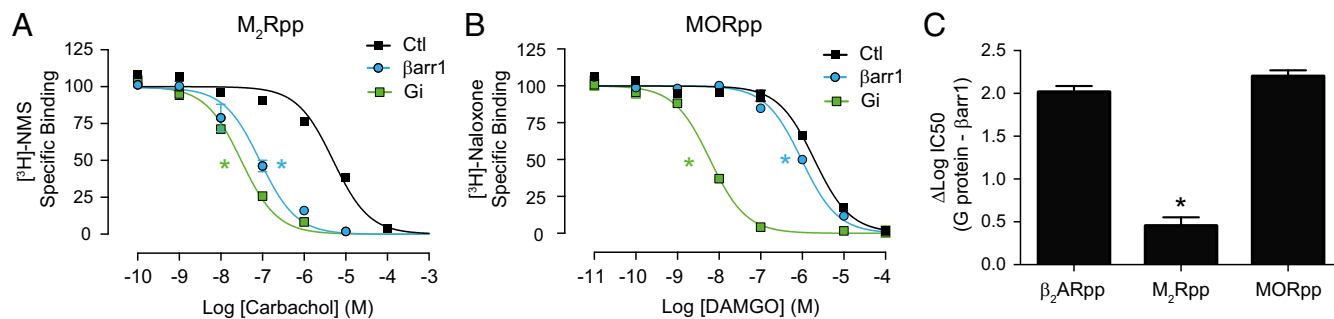
We then asked how this broad range of allostery between GPCRs and  $\beta$ arr might affect the stability and function of these complexes. The ternary complex model posits that the observed enhancement of agonist affinity in the presence of  $\beta$ arr must be reciprocated by an equivalent increase in  $\beta$ arr's affinity for the receptor transmembrane core (11). Thus, the strength of  $\beta$ arr engagement with the receptor core would be expected to follow the same rank order of allosteric cooperativity among the three receptors tested. We assessed the degree of  $\beta$ arr1 engagement by site-specifically labeling its finger loop with monobromobimane ( $\beta$ arr1-bimane); coupling to a receptor's TM core results in an increase in fluorescence due to reduced solvent exposure of the label (19, 20). As expected,  $\beta$ arr1-bimane fluorescence increases for  $\beta_2$ ARpp stimulated with isoproterenol compared with the antagonist ICI-118,551 (Fig. 6A and Fig. S4A). Importantly, the

single domain antibody Nb80, which binds to agonist-activated  $\beta_2$ AR in the same region as G protein, competitively blocks the agonist-induced increase in fluorescence (Fig. 6A). This confirms that the agonist effects on  $\beta$ arr1-bimane are indeed mediated through interaction with  $\beta_2$ ARpp's TM core. Comparison of  $\beta$ arr1-bimane's response to agonist stimulation of  $\beta_2$ ARpp,  $M_2$ Rpp, and MORpp shows that  $M_2$ Rpp displays the highest level of agonist-induced  $\beta$ arr1-bimane engagement (Fig. 6B and Fig. S4), consistent with the observed allosteric cooperativities of these receptors.

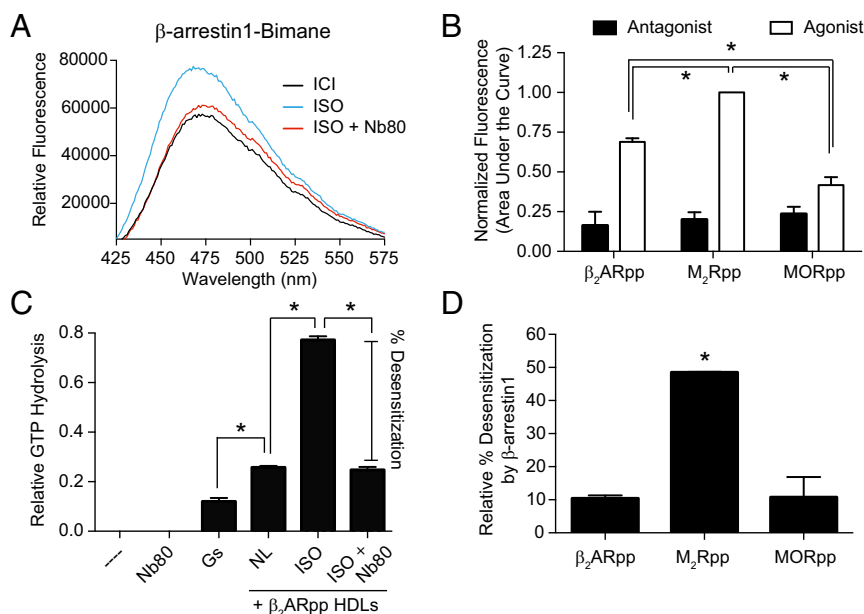
One mechanism by which  $\beta$ arr desensitizes receptors' activation of G protein signaling is steric occlusion of the TM receptor core. We hypothesized that  $\beta$ arr might more efficiently desensitize GPCRs such as the  $M_2$ R—those for which  $\beta$ arr has similar allosteric binding properties as G protein—compared with GPCRs with very divergent transducer coupling, such as the  $\beta_2$ AR and the MOR. To test this, we utilized an in vitro GTPase activity assay that can quantitatively measure agonist-induced receptor activation of G protein. As seen in Fig. 6C, addition of isoproterenol to the  $\beta_2$ AR enhances G protein activation as measured by an increase in GTP hydrolysis, which is blocked by competitive binding of Nb80. A similar agonist-induced increase in GTPase activity is observed for both  $M_2$ Rpp and MORpp (Fig. S5). Desensitization, or inhibition of GTP hydrolysis, by  $\beta$ arr is significantly elevated for  $M_2$ Rpp compared with MORpp and  $\beta_2$ ARpp (Fig. 6D and Fig. S5). Taken together, we find that the efficiency of  $\beta$ arr-mediated receptor desensitization in vitro correlates with the strength of the receptor's allosteric interaction with  $\beta$ arr relative to G protein.

## Discussion

Extensive biochemical and biophysical data indicate that  $\beta$ arr interacts with two distinct GPCR epitopes—a relatively high-affinity interaction with GRK-phosphorylated residues in the receptor C-terminal tail (and/or ICL3) and a much lower affinity interaction with the agonist-activated TM core (reviewed in ref. 21). A number of studies have demonstrated that the former interaction alone induces conformational changes in  $\beta$ arr sufficient to allow it to carry out some of its canonical receptor-dependent functions (10, 15, 16). These include interactions that regulate intracellular signaling cascades (e.g., Src kinase) and facilitate receptor internalization (e.g., AP2 and clathrin). The precise functional contribution of  $\beta$ arr's interaction with the receptor TM core has been more difficult to define due to its dependence on the phosphorylation-dependent conformational changes in  $\beta$ arr. However, multiple lines of evidence argue for its central role in desensitizing G protein-mediated signaling by interacting with the TM core in a mutually exclusive manner (22).



**Fig. 5.** The allosteric enhancement of agonist binding induced by  $\beta$ -arrestin1 varies among different receptors. (A) Competition binding experiments with sortase-ligated  $M_2$ Rpp HDLs, using  $[^3\text{H}]\text{-N}$ -methyl-scopolamine (NMS) as the tracer. Heterotrimeric Gi (100 nM,  $\text{log IC}_{50}$ :  $-7.51 \pm 0.06$ ) and  $\beta$ -arrestin1 ( $\beta$ arr1) (1  $\mu\text{M}$ ,  $\text{log IC}_{50}$ :  $-7.06 \pm 0.08$ ) increase the affinity of the agonist carbachol to a similar extent (no transducer,  $\text{log IC}_{50}$ :  $-5.31 \pm 0.09$ ). (B) Competition binding experiments with sortase-ligated MORpp HDLs, using  $[^3\text{H}]\text{-naloxone}$  as the tracer. Gi (1  $\mu\text{M}$ ,  $\text{log IC}_{50}$ :  $-8.22 \pm 0.05$ ) increases the affinity of the agonist DAMGO to a far greater extent than  $\beta$ arr1 (1  $\mu\text{M}$ ,  $\text{log IC}_{50}$ :  $-6.02 \pm 0.05$ ) (no transducer,  $\text{log IC}_{50}$ :  $-5.71 \pm 0.06$ ). Data in A and B are the mean of three independent experiments, with error bars representing SE, and asterisks (\*) indicate a  $\text{log IC}_{50}$  value significantly different from the control curve ( $P < 0.05$ , one-way ANOVA). (C) Comparison of the difference in agonists'  $\text{log IC}_{50}$  values in the presence of their cognate G proteins versus  $\beta$ arr1 for sortase-ligated  $\beta_2$ ARpp (Fig. 3C),  $M_2$ Rpp (A), and MORpp (B) HDLs.



**Fig. 6.** The extent to which  $\beta$ -arrestin1 engages receptors' transmembrane cores varies among different receptors. (A) Fluorescence spectra of  $\beta$ -arrestin1 ( $\beta$ arr1) labeled with monobromobimane at residue 70 in the finger loop. Activation of HDL- $\beta_2$ ARpp by the agonist isoproterenol (ISO) increases  $\beta$ arr1-bimane fluorescence, which is blocked by Nb80 binding to the receptor TM core. Data shown are representative of three independent experiments. (B) Comparison of  $\beta$ arr1-bimane fluorescence by agonist activation of  $\beta_2$ ARpp,  $M_2$ Rpp, and MORpp HDLs. The area under the fluorescence emission spectra were determined and normalized to  $M_2$ Rpp plus iperoxo (the maximum signal) in each experiment (see Fig. S4 for representative spectra). All three receptors are significantly different from one another ( $*P < 0.05$ ), and  $\beta_2$ ARpp and  $M_2$ Rpp are significantly different from their respective antagonist controls (not indicated,  $P < 0.05$ ). (C) An in vitro GTPase assay measuring GTP hydrolysis as a readout of G protein activation. The basal level of GTP hydrolysis induced by G protein is robustly increased by HDL- $\beta_2$ ARpp HDLs in the presence of ISO compared with no ligand (NL) ( $*P < 0.05$ ), which is blocked (desensitization) by the addition of Nb80 (no significant difference between NL and ISO + Nb80). (D) Inhibition (percentage desensitization) of G protein activation by  $\beta$ arr1 is strongest at  $M_2$ Rpp and significantly different from  $\beta_2$ ARpp and MORpp HDLs ( $*P < 0.05$ ) (see also Fig. S5). Data in B–D are the mean of at least three independent experiments, with error bars representing SE;  $P$  values were determined by one-way ANOVA.

In this study, we circumvented the variable of phosphorylation by using the sortase enzyme to ligate a synthetic phosphopeptide onto the C termini of receptors. Chemical and enzymatic ligation methods have long been pursued as routes to incorporate chemically defined, homogeneous posttranslational modifications into proteins (23, 24), and the sortase enzyme in particular has been used to introduce several types of common modifications, including lipids (25) and glycans (26). Our results demonstrate that sortase has untapped potential to define the contributions of complex posttranslational modification patterns for a variety of protein classes, including phosphoproteins. Our proof-of-principle experiments with three receptors—the  $\beta_2$ AR, the  $M_2$ R, and the MOR—suggest that sortase ligation will be a versatile, generally applicable method to introduce defined phosphorylation patterns into GPCRs and to form GPCR- $\beta$ arr complexes. Interestingly, the  $M_2$ R's native C terminus ends immediately after helix 8 and lacks phosphorylatable residues; all potential GRK phosphorylation sites are found in its particularly large ICL3. Nevertheless, appending the  $V_2$ R-derived phosphopeptide to the  $M_2$ R C terminus is sufficient to promote  $\beta$ arr's interaction with the TM core as assessed by pharmacological, biophysical, and functional measures. In addition, the relative magnitudes of allosteric cooperativity of sortase-ligated  $M_2$ Rpp and  $\beta_2$ ARpp with  $\beta$ arr match well with those previously observed for in cellulose phosphorylated native receptors in membranes (12). This suggests that, at least in some cases, the proximity and activation state of  $\beta$ arr may be more crucial than its precise orientation in promoting its binding to GPCRs' TM core.

Verifying the efficiency and pattern of phosphorylation for GPCRs is technically challenging. As a result, it has been difficult to ascertain whether particular structural elements directly affect GPCRs' interactions with  $\beta$ arr or indirectly influence them at the level of GRK phosphorylation. Sortase-ligated receptors provide a tool to separate these variables, enabling independent

manipulation of phosphorylation state and  $\beta$ arr binding to clarify some of these outstanding questions. For example, our observation that an ICL3 deletion mutant of  $\beta_2$ ARpp still efficiently couples to  $\beta$ arr1 in both radioligand binding and bimane assays conflicts with a previous report that this mutant's interaction with  $\beta$ arr1 is severely impaired (16). However, as the earlier work utilized in cellulose, GRK-phosphorylated receptor, this discrepancy suggests that the ICL3's primary effect might occur at the level of GRK recognition and phosphorylation of agonist-bound receptors.

Mass spectrometry analysis of several receptors has demonstrated that GPCR phosphorylation in cells is quite heterogeneous (27–29). Variation in the stoichiometry and pattern of phosphorylation is observed as a function of external factors such as cell type and the nature of the stimulus, but even individual receptors in the same cell can exhibit different phosphorylation. Since the degree and positioning of phosphorylated residues may affect  $\beta$ arr's activation state, previously described as the “barcode” hypothesis (30), phosphorylation provides a potential mechanism for diversifying a receptor's signaling outcomes. Ultimately we envision using sortase to ligate various phosphopeptides to systematically analyze how specific phosphorylation patterns affect  $\beta$ arr's active conformation, its allosteric interactions with GPCRs, and its interplay with G proteins and downstream signaling effectors.

In this initial study, we ligated the same well-characterized phosphopeptide onto three different receptors, allowing us to detect differences in how  $\beta$ arr interacts with the TM core when activated in the same manner. These three receptors exhibited large variations in their allosteric cooperativity with  $\beta$ arr1, spanning two orders of magnitude, but very similar cooperativities with their cognate G proteins. This observation accords well with the theory that G proteins and  $\beta$ arrs have distinct conformational preferences, providing mechanistic grounds for biased signaling. In the future, sortase-ligated GPCRs could

provide a valuable tool to dissect the molecular basis of how biased agonists differentially couple to transducers.

Our data indicate that  $\beta$ arr cannot harness the full allosteric potential of all GPCRs; receptors' conformational ensembles can inherently bias them toward or against  $\beta$ arr coupling. These differences might be important for the physiological function of GPCRs. Indeed, we found that  $\beta$ arr1 could inhibit G protein activation induced by  $M_2Rpp$  in vitro more effectively than that induced by  $\beta_2ARpp$  or  $MORpp$ , correlating with the strength of the allosteric coupling of the two transducers at each receptor. This variance occurred even under conditions where  $\beta$ arr1 was activated by the same phosphopeptide and was present at a very high effective concentration, due to its high affinity for the ligated C terminus.

It is interesting to speculate how  $\beta$ arr's ability to interact with the GPCR TM core factors into the complex picture of transducer regulation in the cell. In addition to sterically blocking G protein binding,  $\beta$ arrs regulate G protein-mediated receptor signaling by interacting with AP2 and clathrin to promote receptor internalization. Internalized receptors can either be recycled to the plasma membrane or targeted to lysosomes for degradation (22). The choice of pathways is governed by the receptor's phosphorylation, with weaker "class A" patterns that transiently bind  $\beta$ arr favoring recycling and "class B" patterns that stably bind  $\beta$ arr favoring degradation pathways (31). A more recent twist is that G protein activation from intracellular compartments, such as endosomes and Golgi, has now been demonstrated for several receptors, including the  $\beta_2AR$  (32–34). One could hypothesize that the relative strengths of  $\beta$ arr's interactions with the phosphorylated tail and TM core could be sufficient to direct the receptor's fate at multiple junctions. Receptors with class A phosphorylation and weak TM core interactions would desensitize the most slowly, perhaps primarily through negative feedback mechanisms resulting from G protein-mediated

pathways. Class A receptors with stronger TM core interactions would be desensitized primarily by  $\beta$ arr's blockade of the G protein binding site. Class B receptors with weak TM core interactions could continue to activate G proteins even during endocytosis since  $\beta$ arr would not effectively compete, but those with stronger TM core interactions would be silent in G protein signaling as they transit to lysosomes.

It should be noted that most cell-based assays used to study the interaction between  $\beta$ arr and GPCRs are recruitment assays, which will primarily reflect GRK phosphorylation rather than  $\beta$ arr's full engagement of the receptor through the TM core. In fact, the C termini of GPCRs are often replaced with those of class B receptors to enhance the signal. Caution may need to be exercised in using such systems to draw conclusions about the deeply interwoven regulation of GPCRs by G proteins, GRKs, and  $\beta$ arrs. A further understanding of the interaction of  $\beta$ arr with the receptor TM core and its physiological role in GPCR regulation may reveal additional levels of bias which can be targeted in the next generation of GPCR drugs.

## Materials and Methods

Complete details and descriptions of molecular biology methods, protein expression and purification, HDL reconstitution, sortase ligation reactions, bimane fluorescence assays, coimmunoprecipitation experiments, radioligand binding assays, and GTPase assays are provided in *SI Materials and Methods*.

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