

Beta-Arrestin2 as a Competitor for GRK2 Interaction with the GLP-1 Receptor upon Receptor Activation

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Key Words

Bioluminescence resonance energy transfer •
G-protein-coupled receptors • β arrestin •
G-protein-coupled receptor kinase • Glucagon-like peptide 1

Abstract

The signaling of seven transmembrane receptors/G-protein-coupled receptors (GPCRs) is regulated by a number of receptor interacting proteins, including β arrestins (β arrestins) and GPCR kinases (GRKs). In the present report, we have analyzed the interaction pattern between the glucagon-like peptide-1 (GLP-1) receptor (GLP-1R), β arr2, and GRK2 using bioluminescence resonance energy transfer assays. We found that β arr2 interacts with the GLP-1R in a biphasic manner with a phosphorylation-independent and a phosphorylation-dependent component. In competition experiments, we observed β arr2 competing with GRK2 for interaction with GLP-1R. We propose a model where β arr2 competes with GRK2 for interaction with the activated and GRK phosphorylated GLP-1R, suggesting a new role of β arr2 in regulating the orchestration of GRK2 functionality.

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Introduction

Seven transmembrane (7TM) receptors, also referred to as G-protein-coupled receptors (GPCRs), function by initiating intracellular signals as a response to the binding of an agonist to the extracellular and/or transmembrane region of the receptor. This ultimately gives rise to a physiological response which depends on the nature of the receptor and the location of its expression. The fidelity of the cellular and ultimately the physiological response to receptor activation is dependent on a high degree of biological regulation of the receptor-initiated signal. Several proteins interact directly with the receptor to regulate its activity, including β arrestins (β arrestins) and GPCR kinases (GRKs). Of the seven GRKs so far described, four are ubiquitously expressed in mammalian tissues. These are the cytosolic GRK2 and GRK3, and the membrane-attached GRK5 and GRK6. GRKs specifically recognize and phosphorylate the activated receptor, thereby promoting recruitment of cytosolic β arrestins [1]. By interacting with the same domains of the receptor as the G protein, β arrestins sterically prevent further G protein interaction, and thus terminate G protein signaling [2]. Upon binding to an activated receptor,

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β arrs can initiate alternative signaling pathways by acting as a scaffold for a number of proteins [3]. Furthermore, the receptor-bound β arrs can interact directly with clathrin-coated pit components, and thereby mediate internalization of the receptor [4, 5]. In the present report, we have analyzed interaction of the glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) with cytosolic GRK2 and β arr2 using a bioluminescence resonance energy transfer (BRET) assay. BRET is a proximity-based assay that is highly suitable for measuring biological interactions in living cells [6–8]. The GLP-1R is a family-B 7TM receptor which is activated by the peptide hormone GLP-1 and signals through $G\alpha_s$ to mediate the production of cAMP [9]. As the roles of GLP-1 include stimulation of insulin release and reduction of appetite, the GLP-1R is an attractive target for the treatment of type 2 diabetes and obesity [10]. In this report, we demonstrate that β arr2 recruitment to the activated GLP-1R contains both a low-affinity phosphorylation-independent and a high-affinity phosphorylation-dependent component. Furthermore, we demonstrate that GRK2 is recruited to the activated GLP-1R and that β arr2 competes with GRK2 for receptor interaction. Hence, the role of β arr2 in regulating GRK2 interaction with the GLP-1R has strong analogy to its initially discovered function in terminating G protein interaction with the receptor.

Materials and Methods

Molecular Biology

Human β arr2 N-terminally tagged with GFP² (GFP²- β arr2) and Renilla Luciferase (RLuc) cDNA were purchased from PerkinElmer Life and Analytical Sciences (Waltham, Mass., USA). Human GRK2 and GLP-1R cDNA were cloned from a pancreatic cDNA library (Clontech, Mountain View, Calif., USA). C-terminally GFP² or RLuc fusions of the GLP-1R (GLP-1R-GFP² or GLP-1R-RLuc) as well as C-terminally GFP² tagged GRK2 (GRK2-GFP²) and all mutated constructs were generated using standard molecular biology methods. The GLP-1R(tail mut)-RLuc construct contains the following mutations: S431A; S432A; T440A; S441A; S442A; S444A; S445A; S451A; S452A. All cDNA clones were verified by sequencing. Non-coding pcDNA 3.1+ obtained from Invitrogen was used as 'empty vector' in experiments implying so. The agonist GLP-1(7–36)amide used for activation of GLP-1R was obtained from Bachem (Bubendorf, Switzerland).

Transfections and Tissue Culture

cDNAs were transiently expressed in HEK293 cells transfected by calcium phosphate precipitation according to previously reported methods [11]. HEK293 cells were obtained from the European Collection of Animal Cell Cultures. The cells were routinely maintained and passaged as recommended by the supplier.

BRET² Assays

BRET² measurement was done using a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany) as earlier described [12]. All measurements were made at room temperature. For kinetic studies, variable reading times were used and agonist was added with an injector. For BRET² saturation experiments, a range of transfections were made with a stable amount of GLP-1R-RLuc cDNA and increasing amounts of cDNA encoding the GFP²-fused protein. Reading times corresponding to the maximum BRET signal in the kinetic measurements of the different GLP-1R-RLuc constructs were used in the saturation experiment of β arr2 interaction with GLP-1R. The background signal from RLuc was determined by co-expressing the GLP-1R-RLuc with empty vector, and the BRET² ratio generated from this transfection was subtracted from all other BRET² ratios. Expression levels of RLuc- and GFP²-tagged constructs for each BRET² saturation experiment were monitored by luminescence and fluorescence measurements. Luminescence was measured on the Mithras LB 940 plate reader 2 s after Coelenterazine 400A (PerkinElmer Life and Analytical Sciences) addition. For fluorescence measurements, cells from the same transfections were plated in a black clear-bottom microplate (ViewPlate; PerkinElmer Life and Analytical Sciences) at a density of $\sim 5 \times 10^4$ cells per well. After 1 h incubation in darkness, the total fluorescence was measured using the NovoStar microplate reader (BMG LabTech, Offenburg, Germany), with an excitation line at 485 nm and an emission filter at 520 nm. Background values obtained with cells transfected with GLP-1R-RLuc but no GFP² construct were subtracted, and the means of quadruplicate wells/sample were then calculated.

Data Analysis

Nonlinear regression analysis was made using Prism (GraphPad Software, Inc., San Diego, Calif., USA). BRET² values were determined by one-phase exponential association or two-phase exponential association using Prism.

Results

To measure BRET² between the GLP-1R and β arr2 or GRK2, we tagged the GLP-1R C-terminally with RLuc (GLP-1R-RLuc), and both β arr2 and GRK2 with GFP² (N-terminally tagged GFP²- β arr2 and C-terminally tagged GRK2-GFP²). We used GFP² which is a blue shifted variant of GFP developed for the BRET² variant of the BRET assay [13]. In previous reports, we have demonstrated the functionality of the tagged constructs and the suitability of the BRET² assay for measuring β arr2 and GRK2 interaction with the GLP-1R [12, 14, 15]. Using a BRET² assay, we have measured agonist-induced β arr2 recruitment to the GLP-1R with and without co-expression of GRK2, and found that co-expression of GRK2 enhances β arr2 recruitment to the activated GLP-1R (data not shown). In addition, the ability of GRK2 to attenuate GLP-1R-mediated cAMP accumu-

lation due to $G\alpha_s$ uncoupling has been demonstrated previously [14]. Thus, the effect of GRK2 on GLP-1R G protein signaling and β arr2 interaction is consistent with the classical paradigm of GRK2 as a promoter of β arr2 recruitment and subsequent G protein uncoupling from 7TM receptors.

To analyze β arr2 interaction with the GLP-1R in more detail, we performed BRET² time course experiments for agonist-induced recruitment of β arr2 to the GLP-1R. We observed the recruitment of β arr2 to the activated receptor to progress in two phases (fig. 1a). An initial transient peak with maximum after 15–30 s was followed by a steady increase in the BRET² signal reaching a maximum after 1.5–2 min, which was sustained for at least 5 min (fig. 1a). Interaction of the β 2-adrenergic receptor with β arr2 has been demonstrated to consist of both a phosphorylation-dependent and a phosphorylation-independent component [16], with the phosphorylation-independent component constituting a fast initial recruitment of β arr2. We therefore speculated that the transient early peak in recruitment of β arr2 to the GLP-1R reflected phosphorylation-independent β arr2 recruitment. For further analysis, we constructed a GLP-1R-RLuc mutant where all potential GRK phosphorylation sites (Ser/Thr duplets or triplets) in the receptor C-terminal tail were mutated to Ala [GLP-1R(tail mut)-RLuc]. When measuring agonist-induced β arr2 recruitment to the GLP-1R(tail mut)-RLuc mutant, we observed a small transient increase in the BRET² signal corresponding to the transient early peak for β arr2 interaction with GLP-1R observed when using GLP-1R-RLuc (fig. 1b). The more massive and prolonged agonist-induced recruitment of β arr2 observed with the wild-type receptor was not seen with the mutant (fig. 1b). This suggests that the fast and transient peak in the BRET² signal reflects phosphorylation-independent and that the prolonged signal reflects phosphorylation-dependent recruitment of β arr2 to the GLP-1R.

For more detailed analysis we performed BRET² saturation experiments. In these experiments, the amount of the GFP²-fused protein is gradually increased relative to a steady amount of the RLuc-fused protein and the half maximal saturation ratio, or BRET50 value, is a measure of affinity in arbitrary units [7, 15, 17, 18]. Thus, BRET² saturation experiments are suitable for comparing relative affinities for different interaction partners or comparing relative affinities between the different phases of a biphasic interaction. When performing BRET² saturation experiments with GLP-1R-RLuc and GFP²- β arr2, we observed that the BRET² signal, after agonist addition,

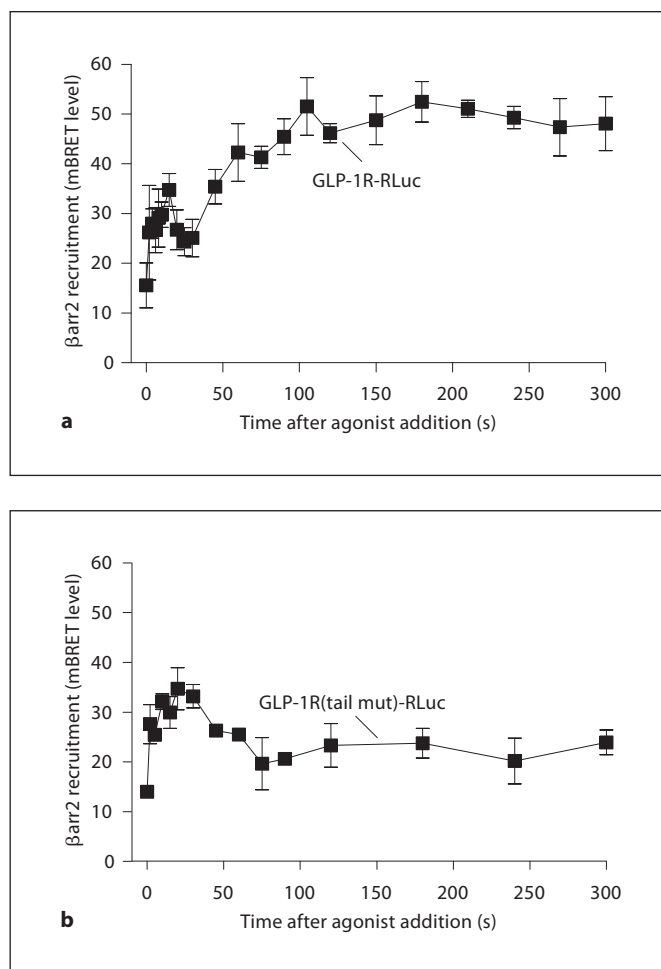


Fig. 1. Time course of agonist-induced β arr2 recruitment to the GLP-1R measured by BRET². **a** Time course of GFP²- β arr2 interaction with GLP-1R-RLuc in transfected HEK293 cells following the addition of 100 nmol/l GLP-1. **b** Time course of GFP²- β arr2 interaction with GLP-1R(tail mut)-RLuc in transfected HEK293 cells following the addition of 100 nmol/l GLP-1. Results are the means $\pm$ SEM of duplicate determinations in three independent experiments.

describes a biphasic saturation curve as a function of the GFP²/RLuc ratio (BRET50 values of 0.18 and 0.016 for the two phases, respectively; fig. 2). This could represent phosphorylation-independent and phosphorylation-dependent interactions, respectively. To potentially isolate a phosphorylation-independent interaction component, we performed saturation experiments with the GLP-1R(tail mut)-RLuc mutant and GFP²- β arr2. In this case, we observed the BRET² signal as describing a monophasic saturation curve as a function of the GFP²/RLuc ratio with a BRET50 value of 0.25 (fig. 2). This further supports

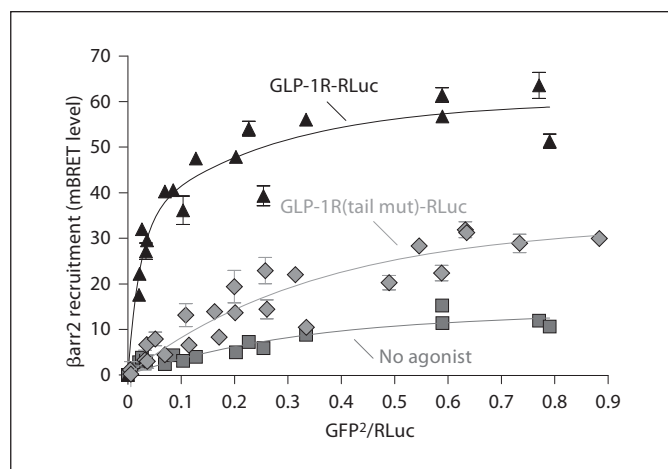


Fig. 2. BRET² saturation experiments for β arr2 interaction with the GLP-1R or GLP-1R(tail mut). Measurements were made on HEK293 cells from different transfections expressing an increasing amount of GFP²- β arr2 relative to a steady amount of GLP-1R-RLuc or GLP-1R(tail mut)-RLuc. Measurements were made without stimulation (no agonist) or after 5 min (GLP-1R-RLuc) or 20 s [GLP-1R(tail mut)-RLuc] of stimulation with 100 nmol/l GLP-1. The curve shown in the figure for no agonist addition is made with GLP-1R-RLuc. Data are pooled results from three independent experiments each carried out in quadruplicates.

that the high-affinity phase, but not the low-affinity phase, of receptor interaction with β arr2 is phosphorylation-dependent.

We proceeded by characterizing GLP-1R interaction with GRK2. When measuring agonist-induced recruitment of GRK2-GFP² to GLP-1R-RLuc, we observed a relatively fast increase in the BRET² signal which peaked at ~30–40 s before reaching a lower plateau (fig. 3a). The investigation of β arr2 recruitment to GLP-1R presented in figures 1 and 2 was performed in the presence of endogenous and presumably limiting levels of GRK2 in the presence of GLP-1R overexpression, and is thus not suitable for comparison to the investigation of recruitment of exogenous levels of GRK2 to GLP-1R as shown in figure 3a. Thus, to compare with β arr2 interaction with GLP-1R under conditions of exogenous GRK2 expression, we measured GFP²- β arr2 recruitment to the activated GLP-1R-RLuc after co-expression of GRK2 (fig. 3b). We observed the BRET² signal to peak at ~30–40 s after which it steadily declined (fig. 3b). Thus, there is an apparent overlap in the time course of GRK2 and β arr2 interaction with the activated GLP-1R under conditions of exogenous GRK2 expression. When performing BRET² saturation experiments with GLP-1R-RLuc

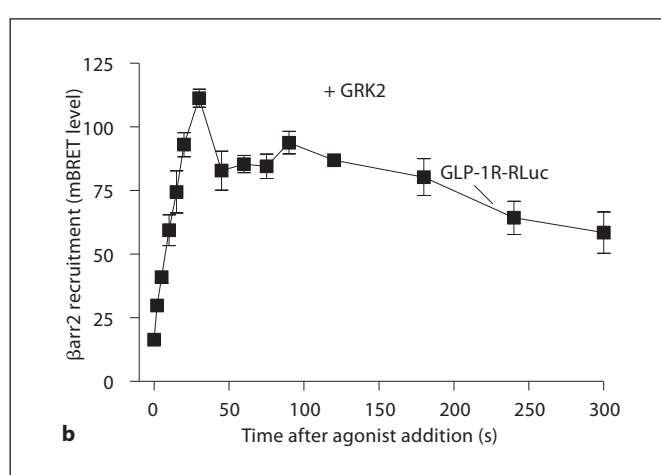
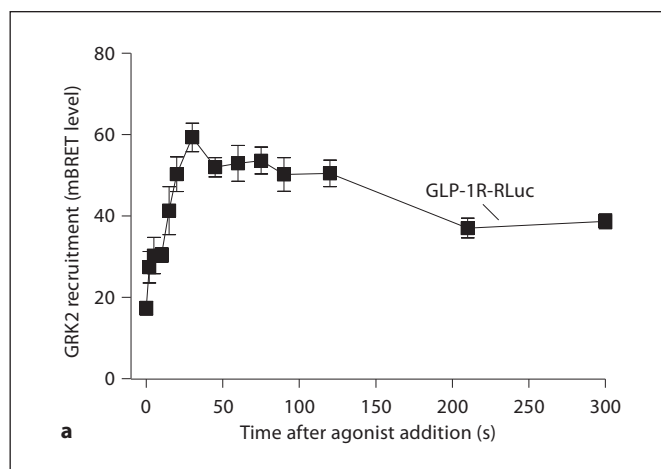


Fig. 3. Time course of agonist-induced GRK2 or β arr2 recruitment to the GLP-1R measured by BRET². **a** Time course of GRK2-GFP² interaction with GLP-1R-RLuc in transfected HEK293 cells following the addition of 100 nmol/l GLP-1. **b** Time course of GFP²- β arr2 interaction with GLP-1R-RLuc in transfected HEK293 cells following the addition of 100 nmol/l GLP-1. Untagged GRK2 was co-expressed to mimic the conditions of increased GRK2 activity in **a**. Results are the means $\pm$ SEM of duplicate determinations in at least three independent experiments.

and GRK2-GFP², we observed the BRET² signal to describe a monophasic saturation curve as a function of the GFP²/RLuc ratio with a BRET₅₀ value of 0.14 (fig. 4). This corresponds to an approximately 10-fold lower affinity compared to the BRET₅₀ value for the high-affinity phase of the β arr2 interaction with GLP-1R (fig. 2). Given the overlapping time course of GRK2 and β arr2 recruitment to the activated GLP-1R and the observation that the activated receptor displays 10-fold higher affinity for β arr2 compared to GRK2, we speculated

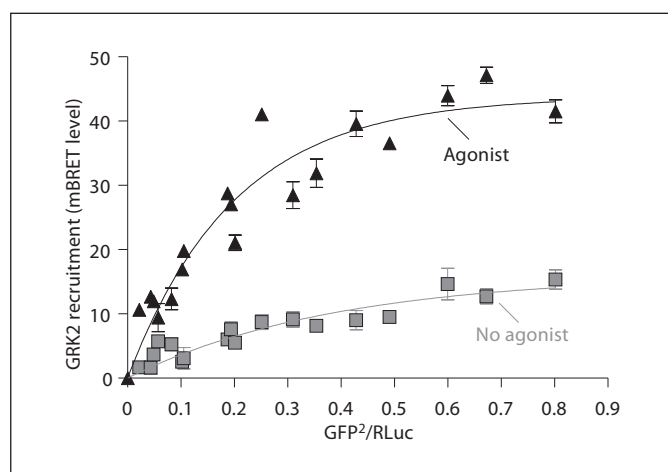


Fig. 4. BRET² saturation experiments for GRK2 interaction with the GLP-1R. Measurements were made on HEK293 cells from different transfections expressing an increasing amount of GRK2-GFP² relative to a steady amount of GLP-1R-RLuc. Measurements were made without stimulation (no agonist) or after 5 min of stimulation with 100 nmol/l GLP-1 (agonist). Data are pooled from three independent experiments each carried out in quadruplicates.

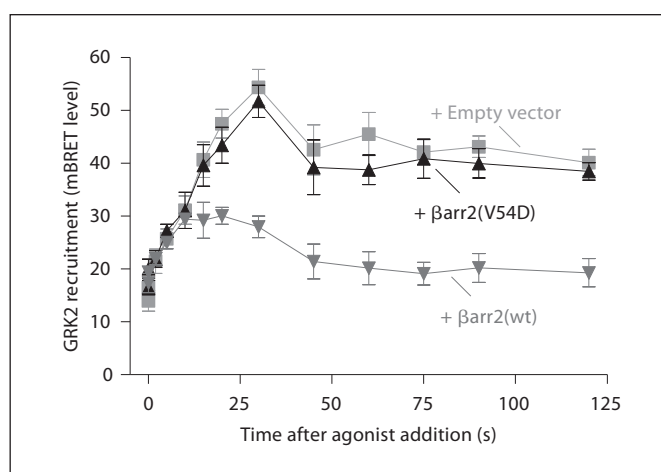


Fig. 5. BRET² competition experiments. Time course of GRK2-GFP² interaction with GLP-1R-RLuc after addition of 100 nmol/l GLP-1. HEK293 cells were co-transfected with cDNA encoding either untagged β arr2(wt), β arr2(V54D) or empty vector to analyze for the ability to compete with GRK2-GFP² for GLP-1R-RLuc interaction. Results are the means $\pm$ SEM of duplicate determinations in six independent experiments.

that β arr2 could have a role in displacing GRK2 from the receptor. To analyze this, we performed BRET² time course experiments for agonist-induced interaction of GRK2-GFP² with GLP-1R-RLuc upon co-expression of either empty vector, β arr2(wt), or a β arr2(V54D) mutant lacking the ability to interact with the receptor [19] (fig. 5). The lack of interaction between β arr2(V54D) and GLP-1R was confirmed in a BRET² assay where no recruitment of GFP²- β arr2(V54D) to agonist-activated GLP-1R-RLuc was observed (data not shown). In contrast to the prolonged agonist-induced interaction of GRK2-GFP² with GLP-1R-RLuc observed upon co-expression of empty vector, GRK2-GFP² interaction was transient and the maximum BRET² signal severely reduced when β arr2(wt) was co-expressed (fig. 5). On the contrary, the non-receptor binding β arr2(V54D) mutant had no effect on GRK2-GFP² interaction with GLP-1R (fig. 5). This β arr2-mediated competition of GRK2 interaction with the GLP-1R is not caused by changes in the expression level of the receptor or GRK2 upon co-expression of β arr2 since co-expression with neither β arr2(wt) nor β arr2(V54D) changed the GRK2-GFP² / GLP-1R-RLuc expression ratio (online suppl. fig. 1, see www.karger.com/doi/10.1159/000330742).

Discussion

In the present report, we characterized the details of GLP-1R interaction with β arr2 and GRK2. When analyzing the time course of GLP-1R interaction with β arr2, we found agonist-induced β arr2 recruitment to the receptor to progress through two phases – a minor rapid and transient phase followed by a relatively massive and sustained phase, which we suggest represents phosphorylation-independent and phosphorylation-dependent β arr2 interactions, respectively. 7TM receptor C-terminal tail Ser/Thr doublets or triplets are potential GRK phosphorylation sites and determinants for β arr2 interaction [20], and agonist-induced phosphorylation of the GLP-1R has been demonstrated to be restricted to the C-terminal tail Ser/Thr doublets [21]. By using a GLP-1R mutant with all C-terminal Ser/Thr doublets and triplets mutated to Ala, we could isolate the presumably phosphorylation-independent β arr2 interaction with GLP-1R. BRET² saturation experiments with the GLP-1R and β arr2 revealed a biphasic interaction, and results with the GLP-1R(tail mut) mutant further supported that the low-affinity phase of GLP-1R interaction with β arr2 was phosphorylation-independent and the high-affinity phase was phosphorylation-dependent. Thus, the applied BRET² saturation experimental approach presented an opportunity to

compare the effect of GLP-1R phosphorylation on receptor affinity for β arr2. The results suggest that phosphorylation of the GLP-1R increases the affinity for β arr2 approximately 10-fold. To our knowledge, this is the first example where the magnitude of phosphorylation promoted increase in affinity for β arr2 has been reported for a family-B 7TM receptor. Our results are consistent with data for the family-A β 2-adrenergic receptor where receptor phosphorylation has been reported to increase the affinity for β arr2 10- to 30-fold in assays with reconstituted receptors and purified β arr2 [22, 23]. It is interesting to note the similarity of the reported values despite the use of different receptors and different experimental approaches. Given that the initial phosphorylation-independent recruitment of β arr2 to the activated GLP-1R is transient, relatively minor compared to the phosphorylation-dependent recruitment, and observed under conditions of β arr2 overexpression, the functional consequence of this interaction may be modest under normal cellular conditions. The molecular basis for the observed transient phosphorylation-independent β arr2 interaction with the GLP-1R remains undescribed. Yet, based on several studies, β arrs have been proposed to have two binding sites on 7TM receptors: one dependent on agonist-induced conformational changes in the receptor involving primarily ionic interactions which lead to an initial recruitment of the arrestin; and another dependent on receptor phosphorylation involving hydrophobic interactions assumable mediating a more stable arrestin-receptor interaction [24]. This proposal is in agreement with the findings of this study.

In BRET² time course experiments using exogenous levels of GRK2, we observed an apparent overlap in the time course of GRK2 and β arr2 recruitment to the activated GLP-1R. Furthermore, BRET² saturation experiments suggested that the receptor displays 10-fold lower affinity for GRK2 compared to the major phosphorylation-dependent component of β arr2 interaction, which presumably appears subsequent to GRK-mediated receptor phosphorylation. It should be noted that the inherent limitations to the time resolution of the BRET experiments and the fact that we are measuring on a population of receptors rather than single molecules, will not allow us to temporally distinguish GRK2 and β arr2 recruitment to the receptor if occurring within seconds. Given the sizes of GRK2 and β arr relative to 7TM receptors and the assumption that the two proteins are interacting with overlapping domains of the receptor (at the C-terminal tail), it is plausible that there is limited space for simultaneous interaction of GRK2 and

β arr2 with the receptor [25–27]. This led us to speculate that β arr2 competes with GRK2 for receptor interaction following receptor phosphorylation. The sustained BRET² signal observed when measuring GRK2 recruitment to the GLP-1R in the absence of β arr2 co-expression is considered related to the endogenous amounts of β arr2 being insufficient under the assay conditions of GLP-1R and GRK2 overexpression. When wt β arr2 was co-expressed, we observed a significant competition of GRK2 interaction with the GLP-1R resulting in a relatively minor and highly transient recruitment of GRK2 to the activated receptor. The competitive effect of β arr2 was dependent on receptor interaction as a non-receptor interacting β arr2(V54D) mutant had no effect on GRK2 interaction with the receptor. Thus, we suggest that β arr2 competes with GRK2 for interaction with the GLP-1R following GRK2-mediated receptor phosphorylation.

Such a role of β arr2 in competing with GRK2 for receptor interaction would bear strong resemblance to the function of β arr2 in terminating G protein signaling by competing with the G protein for receptor interaction. Within recent years, GRK2 has been demonstrated to have several non-receptor kinase related functions through interactions with a broad range of proteins in both phosphorylation dependent and independent manners, including other membrane proteins, structural proteins, and kinases [28]. In addition, the importance of GRK2 in several pathophysiological conditions has only recently given GRK2 and interaction partners the status as new possible pharmaceutical targets [29]. Hereby, it has become important to describe the mechanism behind the regulation of the GRK2 interactome. Our results suggest β arr2 takes action in this regulation by displacing GRK2 from the receptor after phosphorylation. The kinetics of this mechanism are likely to depend on β arr2 expression under different conditions, in different cell types, local membrane compositions (e.g. microdomains), etc. It may be speculated that a prolonged interaction of GRK2 with the receptor retains GRK2 in a membrane environment with close proximity to several local substrates and interaction partners. Thus, the competition of β arr2 for receptor interaction could potentially play a role in the orchestration of GRK2 function beyond receptor phosphorylation. In relation to this, it is interesting that β arrs have been reported to mediate GRK2 degradation through proteasomal proteolysis by scaffolding e.g. ubiquitin ligases to the ubiquitination of GRK2 upon activation of the β 2-adrenergic receptor [30]. A similar mechanism for degradation of GRK2 af-

ter receptor activation and β arr binding to GLP-1R remains undescribed.

In conclusion, we here demonstrate that β arr2 interaction with the GLP-1R consists of both a phosphorylation-independent and a phosphorylation-dependent phase, and suggest that receptor phosphorylation increases the GLP-1R affinity for β arr2 10-fold. Furthermore, we demonstrate that the phosphorylated receptor affinity for β arr2 is 10-fold higher compared to GRK2, and that β arr2 competes with GRK2 for receptor interaction. Our data suggest that the classical view on the interaction dynamics that follow receptor activation should be expanded to include β arr2 as a regulator of GRK2 interaction with the receptor in addition to its known role in regulating G protein coupling. Future experiments will further reveal the importance of β arr2 in orchestration of the GRK2 interactome.

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Disclosure Statement

All authors except Christian E. Elling are employees of Novo Nordisk A/S and own company stocks.

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