Phosphorylation Uncouples the Gastrin-releasing Peptide Receptor from $G_q^*$

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Previous work on the desensitization of $G$ protein-coupled receptors has focused on the role of arrestin binding following receptor phosphorylation. We have examined the hypothesis that phosphorylation alone contributes to desensitization. In this study we demonstrate that for the $G_q$-coupled gastrin-releasing peptide receptor (GRP-R), phosphorylation by GRK2 to a stoichiometry of $\sim 1$ mol PO$_4$/mol GRP-R is sufficient in the absence of arrestin to reduce the rate of receptor catalyzed $G$ protein activation by approximately 80%. Furthermore, GRP-Rs exposed in vitro to agonist are rapidly phosphorylated to a similar stoichiometry and are desensitized to a similar degree. Finally, the molecular mechanism for both in vitro GRK2-induced and in vivo agonist-induced desensitization is primarily a decrease in the maximum velocity ($V_{\text{max}}$) for the catalysis of guanine nucleotide exchange by the GRP-R rather than a change in the affinity of the receptor for the $\alpha$ or $\beta\gamma$ subunits. Based on these results, we suggest that, for some $G$ protein-coupled receptors, phosphorylation has a role in desensitization that is independent of arrestin.

Bombesin-like peptides elicit a variety of effects including mitogenesis, hormone secretion, and modulation of neuron firing rate (1). These effects are transduced through a family of $G$ protein-coupled receptors (GPCRs) including the gastrin-releasing peptide receptor (GRP-R) (2, 3). An agonist-activated GPCR catalyzes the exchange of guanine nucleotide on the $\alpha$ subunit of a heterotrimeric $G$ protein leading to the formation of $G\alpha$-GTP. The subsequent dissociation of the heterotrimeric $G$ protein leads to stimulation of signal transduction cascades mediated by the free $G\alpha$-GTP and $G\beta\gamma$ subunits (4). In the bombesin receptor family, the activated GRP-R catalyzes guanine nucleotide exchange on $G\alpha_q$ (5) to activate effectors including phospholipase C- $\beta$ (3).

Mechanisms to attenuate receptor signaling have evolved that limit the amplitude and/or duration of the signal transduction cascade(s) and are collectively referred to as “desensitization.” At the molecular level, desensitization may result from the degradation of ligand, from changes in receptor availability or activity, or from changes in the availability or activity of downstream effector molecules (6). Rhodopsin, which signals through transducin ($G_i$), and the $\beta_2$-adrenergic receptor ($\beta_2$AR), which signals through $G_q$, have been the most extensively studied GPCRs. In both cases, rapid agonist-induced receptor phosphorylation along with subsequent binding of an arrestin to the receptor play critical roles in receptor deactivation (7, 8). Many other GPCRs are also known to be rapidly phosphorylated after addition of agonist (9, 10), including the GRP-R (11). Two classes of protein kinase have been implicated in agonist-induced GPCR phosphorylation: 1) the second messenger-dependent protein kinases A and C and 2) the second messenger independent kinases, called G protein-coupled receptor kinases (GRKs), including rhodopsin kinase (RK or GRK1) and the $\beta$-adrenergic receptor kinase 1 ($\beta$ARK1 or GRK2) (12). Several GRKs are known to be substrates for phosphorylation by GRKs in vitro (12).

In vitro reconstitution systems have been used to evaluate the relative contributions of phosphorylation and arrestin for uncoupling. For rhodopsin, RK phosphorylation alone inhibits coupling to $G_i$, and the intrinsic inhibitory effects of phosphorylation on coupling are augmented by the addition of arrestin (13, 14). But for the $\beta_2$AR, neither in vitro phosphorylation by $\beta$ARK (GRK2) (15, 16) nor in vivo desensitization by agonist (17) has a large effect on reconstituted receptor coupling. In contrast, phosphorylation of the $\beta_2$AR by protein kinase A does deactivate the receptor (16), as does addition of $\alpha$-arrestin to $\beta$ARK1-phosphorylated $\beta_2$AR (18). This suggests that for the $\beta_2$AR, GRK-induced phosphorylation serves primarily as a requisite for the binding of $\alpha$-arrestin. Hence, receptor phosphorylation alone appears to have a variable effect upon coupling, depending upon assay conditions, receptor subtype, and protein kinase. But the $\beta_2$AR is often viewed as the best model for GPCR desensitization because it is unclear whether data obtained from the unique environment of the visual system can be extrapolated to any other GPCR.

The GRP-R undergoes rapid, agonist-induced desensitization (3), but the molecular mechanisms governing its desensitization are unknown. The GRP-R is rapidly phosphorylated in vitro by a protein kinase other than protein kinase C after exposure to bombesin (BN) (11), but the role of phosphorylation in desensitization is undefined. Additionally, the molecular events resulting in desensitization of $G_q$-coupled receptors (such as the GRP-R) have been less well studied than for rhodopsin or the $\beta_2$AR. It is unclear whether the mechanisms governing desensitization will be the same for receptors that use different cognate $G$ protein(s). For example, for the muscarinic cholinergic receptors, which can couple to $G_{\text{ia}}$ and $G_q$, differences were found in the agonist- and GRK-induced phosphorylation and desensitization among and between $G_{\text{ia}}$- and $G_q$-preferring receptor subtypes (9, 19). Therefore, to begin examining the molecular mechanisms involved in GRP-R de-
sensitization, this study was undertaken to determine whether the GRP-R is a substrate in vitro for phosphorylation by a GRK, and if so, whether GRK phosphorylation effects GRP-R coupling.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium, fetal bovine serum, G-418, penicillin/streptomycin, and Spin column serum-free medium were from Life Technologies, Inc. Tris-Glycine polyacrylamide gels were from Novex. BN was from Bachem (Torrance, CA). [32P]H3PO4 (30 Ci/mmol) was from ICN. Enucleated frozen eyes from Loligo forbesi (26) were purified and Northup (27) for squid, was supplemented with 10% bovine serum. Spodoptera frugiperda cells (SF9 cells) were maintained in Spin column medium at 27 °C.

**Membrane Preparation and Urea Extraction**—Published methods (5) were used to prepare GRP-R containing membranes from 5 ET4, a Balb/c 3T3 mouse fibroblast cell line expressing a Myc epitope-tagged GRP-R (11), and SF9 cells infected for 48 h with a GRP-R-E4, a Balb/c 3T3 mouse fibroblast cell line expressing a Myc epitope-tagged GRP-R (5, 23) because, in this system, the receptor is never removed from a biologically relevant membrane. Phosphatases in the postnuclear (P2) membranes. 6 M urea-

**Western Blotting**—Samples were resolved on 10% Tris-Glycine polyacrylamide gels, transferred overnight onto nitrocellulose, and Western blotted using the pan-arrestin antibody F4C1 at a dilution of 1:250.

**Quantitation of Ligand Binding Sites**—GRP-R ligand binding sites in the membrane preparations were quantitated by analysis of the displacement of the specific GRP-R antagonist [3H]-GRP

**Purification of G Proteins**—Cuttlefish retinal Go, (26) was purified from cuttlefish (S. officinalis) photoreceptors as described by Hartman and Northup (27) for squid (Loligo forbesi) with one modification: solution C used in the thawing and washing of the photoreceptor membranes was supplemented with 10 μM GDP. Bovine retinal Go and bovine brain Go by (brain Go) (29) were purified using published procedures.

**In Vitro Phosphorylation and Stoichiometry**—Samples were incubated in a solution with 20 mM MOPS, pH 7.5, 3 mM MgSO4, 1 mM EDTA, and 100 μM AEBSF. Other reagents were added as indicated in the text and figures. Inclusion of 1 mM TMP and 100 mM ATP completely inhibited nucleotide activity present in the membranes phosphorylation as determined by evaluating adenosine phosphates with thin layer chromatography with polyethyleneimine cellulose as described previously (data not shown) (30). Reactions were incubated at 30 °C for 30 min. Incorporation of [32P]ATP was assessed by stopping the reaction with an equal volume of 2% SDS then collecting the GRP-R by immunoprecipitation. A modification of previously described procedures (11) was used in which 5 μl of the GRP-R-specific antibody 3 and 30 μl of protein A/G slurry were added per 500 μl of final volume. Immunoprecipitates were resolved on a 4–20% Tris-Glycine polyacrylamide gel, and the gel was fixed and dried and exposed to either x-ray film or a PhosphorImager screen (Molecular Dynamics). Radiospecific activity was determined by spotting a small volume of the reaction solution onto the filter paper with the dried gel and exposing both to the PhosphorImager screen at the optimum PhosphorImager exposure time for each gel. Phosphorylation of GRP-R and reaction mix were determined, and then the phosphorylation stoichiometry was calculated using the number of antagonist binding sites present in the membranes at the end of the assay.

**Determination of in Vivo Stoichiometry of Phosphorylation**—5 ET4 cells were phosphorylated using a modification of previous methods (11). Cells were incubated for 3 h at 37 °C in phosphate-free medium with 50 μCi of [32P]O4/P (PF medium) and then exposed to 1 μM bombesin for 5 min. Reactions were stopped by removing PF medium, washing the cells twice with ice-cold phosphate-buffered saline, and adding 2% SDS/50 mM Tris, pH 8.0. Phosphorylated GRP-Rs were collected by immunoprecipitation as described above. Total intracellular ATP was measured from a replicate dish using the Bioluminescent Somatic Cell Assay Kit (Sigma) according to the manufacturer’s instructions. Whole cell [32P]ATP was determined using adenosine phosphates in a replicate dish with thin layer chromatography with polyethyleneimine cellulose as a modification of previous methods (30). PF medium was removed, and the cells were washed twice with phosphate-buffered saline, and then the mixture was neutralized with 1 M HC03. 2 μl of the supernatant was then applied to a thin layer plate of polyethyleneimine cellulose, and the chromatogram was developed with 0.5 M LiCl in 2% formic acid. The plate was dried, and adenosine phosphates were visualized with an ultraviolet light source. The location of the [32P] was determined by exposing the plate to X-ray film and quantitated with a PhosphorImager. Cell number in a replicate dish was determined, and receptor number on whole cells was measured as described (31).

**In Vitro Coupling Assay (GDP/GTPyS Exchange Assay)**—The GRP-R catalyzed exchange of GDP for GTPyS on Go was determined by modification of published procedures (5). Solution G for all experiments was prepared 50 mM HEPES, pH 7.4, 1 mM EGTA, 150 mM KCl, 5 mM MgCl2, 0.2 mM bovine serum albumin, 1 mM dithiothreitol, and 100 mM NaCl. 0.5–2 μM GDP, 8–16 mM (0.01–0.02 μCi/μl) [35S]GTPyS, and 1 mM TMP were added to solution G as indicated. For two-step phosphorylation/coupling assays, the total reaction volume was 100 μl. Ice-cold solution G was supplemented with other reagents as indicated. Reactions were initiated by adding GRP-R-containing membranes (treated with or without GRK2 in the phosphorylation assay) directly to the other components without any processing. Reaction velocities were determined by incubating samples at 30 °C and removing 10-μl aliquots at timed intervals. Aliquots were added to 4 ml of ice-cold solution S (20 mM Tris-HCl, pH 8, 100 mM NaCl, 25 mM MgCl2) to terminate the reaction, and then samples were processed and radioactivity was quantitated as described.

**One-step coupling assays, reactions were usually carried out in 0.2-ml polymerase chain reaction tubes (Molecular BioProducts, San Diego, CA) with a total reaction volume of 20–50 μl. Membranes containing the GRP-R were mixed on ice with Gb2 and solution G-supplemented as described in the figures. The mixture was prewarmed for 30 s at 30 °C, and then the reaction was initiated by addition of Go. Samples were incubated for the times indicated. In the presence of TMP, all reaction velocities were constant with time for at least 15 min, and the measurement of GTPyS binding at a single time point provided similar results to those obtained using rates obtained from progress curves (data not shown). Vmax and Km were determined using KaleidaGraph (Abelbeck Software) and fitting the data for initial velocities to a single site binding model.

**RESULTS**

**In Vitro Phosphorylation of the GRP-R by GRK2**—We first examined whether the GRP-R is a substrate for phosphorylation by a GRK in vitro. As our source of receptor, we chose to use urea-extracted membranes from transfected mammalian cells expressing high levels (≈550,000/cell) of a Myc epitope-tagged GRP-R (5, 23) because, in this system, the receptor is never removed from a biologically relevant membrane. Phospholipids have been shown to be important in regulation of GRK activity (32), and therefore a relevant membrane environment may be crucial for studying receptor phosphorylation and desensitization. Urea-extracted membranes from SF9 cells infected with a GRP-R containing baculovirus (22) were used for these experiments to confirm that there were no membrane-specific or Myc tag-dependent effects.

Fig. 1 shows the in vitro phosphorylation of the Myc-tagged GRP-R by GRK2 (βARK1). There is no GRP-R phosphorylation in the absence of added GRK2 (lane 2), the GRP-R is a substrate for phosphorylation by GRK2 only in the presence of...
agonist (compare lanes 3 and 6 with lanes 4 and 7), and the GRK2-mediated GRP-R phosphorylation is markedly enhanced by the presence of Gβγ (compare lanes 3 and 6 with lane 8). The presence or absence of GTPγS has no effect on the phosphorylation reaction (compare lanes 3 and 4 with lanes 6 and 7), suggesting that there is no effect of endogenous Gα subunits. These results are consistent with previous data on the requirements for phosphorylation of GPCRs by GRK2 (33). In the presence of 300 nM bovine brain Gβγ, maximum phosphorylation (of 7.2 nm GRP-R) in a 30-min assay occurred with 300 nM GRK2, and the stoichiometry of GRK2-dependent phosphorylation was ~0.9 mol PO4/mol GRP-R detectable by autoradiography (data not shown).

**GRP-R Phosphorylation by GRK2 Uncouples the Receptor from Gq**—To study the effect of phosphorylation on coupling to G proteins, a two-step phosphorylation-coupling-procedure was performed. In these experiments, 7 M urea-extracted GRP-Rs (expressed in either membrane environment) were first incubated with 150 nM bombesin in the presence or absence of GRK2 for 30 min. Bovine retinal Gβγ complex was used to enhance phosphorylation. In the second step, the receptors were incubated in the presence of G protein subunits (Gαq and Gβγ), GDP, and [32P]GTPγS, to assess GRP-R catalyzed guanine nucleotide exchange on Gαq. Cuttlefish retinal Gαq was utilized as a reporter for coupling because the GRK2-couples to this Gαq efficiently (5, 23), and large quantities of cuttlefish Gαq can be prepared. Catalytic activity of the GRP-R without agonist was measured by adding an excess of ME (a pure GRP-R antagonist) to some of the reactions. The ~ME condition is equivalent in the coupling assay to no added bombesin (data not shown).

In Fig. 2, data are presented as a progress curve showing [32P]GTPγS binding as a function of time. In both mouse fibroblast (Fig. 2) and insect cell membranes (not shown), in vitro GRK-2-mediated phosphorylation of the GRP-R uncouples the receptor from Gαq. All experiments performed using these conditions showed no detectable increase in the catalysis of [32P]GTPγS binding by bombesin for GRK2-treated receptors above the level catalyzed by either receptor preparation in the presence of antagonist. The rate of [32P]GTPγS binding catalyzed by control membranes in the presence of bombesin was significantly higher (2.9 ± 0.4 fold, n = 4) than the rate catalyzed by GRK2-treated membranes in the presence of bombesin. The uncoupling is not a result of the selective loss of correctly folded receptor because the number of antagonist binding sites are equivalent in both GRK2-treated and untreated conditions following a 30-min incubation (data not shown). Uncoupling requires the presence of ATP in the phosphorylation step because the replacement of ATP by the non-hydrolyzable analog AMP-PCP prevented GRK2-dependent uncoupling (data not shown). However, the continued presence of ATP is not required because the addition of AMP-PCP after the phosphorylation step did not prevent uncoupling (Fig. 2). Additionally, bombesin is needed in the phosphorylation step for GRK2 to uncouple the GRP-R because the addition of GRK2 without bombesin did not uncouple the GRP-R (data not shown). This implies that uncoupling requires the phosphorylation of the GRP-R because the receptor is not phosphorylated without the addition of agonist (Fig. 1). Finally, phosphorylation-induced uncoupling is a function of the stoichiometry of phosphorylation because partial uncoupling was seen with lower stoichiometry of phosphorylation (data not shown).

**In Vivo Exposure to Agonist Uncouples the GRP-R from G Protein**—To determine the stoichiometry of bombesin-stimulated in vivo GRP-R phosphorylation, 5 ET4 cells were metabolically labeled with [32P]Pi and then exposed to 1 μM bombesin or no added peptide for 5 min. GRP-Rs were collected by immunoprecipitation and total ATP and [32P]Pi determination as described under “Experimental Procedures.” Stoichiometry of
Phosphorylation Uncouples the GRP Receptor from $G_q$

Phosphorylation-induced uncoupling of rhodopsin and the arrestin family proteins have been implicated in agonist-exposure occurring at similar sites. There was no detectable arrestin contamination in brain membranes to control membranes are shown in Fig. 3A. Therefore it is unlikely that retinal GRP-Rs are uncoupled from $G_q$ without detectable arrestin—arrestin is a major factor in the phosphorylation-induced uncoupling observed.

In Vivo Agonist-exposed and In Vitro GRK2-phosphorylated GRP-Rs Are Uncoupled from $G_q$ Without Detectable Arrestin—The experiment using in vivo agonist-exposed GRP-R was repeated with brain $\beta_7$-AR (Figs. 3B and 4A). The brain $\beta_7$ was chosen (for this and subsequent experiments) for several reasons: retinal $\beta_7$ is unlikely to be physiologically relevant for GRP-R coupling, other data have suggested that brain $\beta_7$ fractions enhance GRP-R catalyzed guanine-nucleotide exchange on $G_{q\alpha}$ more efficiently than $\beta_1$ (23), and there was no detectable arrestin contamination in brain $\beta_7$. In vivo agonist-exposed GRP-Rs had about one-fourth the rate of catalysis of guanine-nucleotide exchange in the presence of bombesin and brain $\beta_7$ as did control receptors (26 ± 4%, $n = 10$), similar to the results found with $\beta_1$. Subsequently, the in vitro phosphorylation coupling experiment was repeated with brain $\beta_7$ and two further modifications: 1) the in vitro GRK2-

For all panels, a background is subtracted from each time point consisting of bombesin-treated, GRK2-treated, or control membranes incubated under the same conditions without G protein subunits. Data are representative of at least three experiments (see text).
phosphorylated membranes were urea-extracted (again) following phosphorylation to remove all GRK2 and βγ subunit present in the phosphorylation step, and 2) the concentration of bombesin added in the coupling assay was increased to 1 μM (the same concentration as was used in Fig. 3, A and B). In the presence of 90 nM brain βγ (Fig. 3C) or 300 nM brain βγ (Fig. 4B), GRK2-induced phosphorylation of the GRP-R eliminated approximately 80% of the GRP-R-specific guanine-nucleotide exchange (rate of catalysis 22 ± 5% of control rate, n = 11). These data suggest that with either βγ dimer preparation and without any detectable arrestin-like reactivity in the assay (with brain βγ), GRP-R coupling to Gαq is greatly attenuated by either GRK2 phosphorylation in vitro or agonist exposure in vivo.

In Vitro (GRK2-dependent) and in Vivo (Agonist-dependent) Uncoupling of the GRP-R Occur Primarily via a Decrease in the Vmax for Catalysis of Guanine-Nucleotide Exchange—To determine the molecular mechanism responsible for the decrease in the velocity of catalysis of guanine-nucleotide exchange on Gαq, saturation curves for Gαq, and βγ were performed on both in vitro phosphorylated and in vivo bombesin-exposed GRP-R. Experiments examining Gαq saturation were first performed with equal concentrations of receptors (from 1.4–2 nM) (Fig. 4). In all experiments a decrease in rate of catalysis for the GRK2- and agonist-treated receptors was seen with all concentrations Gαq tested, consistent with the data shown in Figs. 2 and 3. After subtraction of uncatalyzed binding of [35S]GTPγS to Gαq alone, Kmax and Vmax values could be determined for control and agonist-pretreated GRP-Rs. Kmax values (mean ± S.D.) for control (210 ± 51.4 nM, n = 6) and agonist-pretreated receptors (282 ± 72 nM, n = 3) were similar, whereas the Vmax for the agonist-pretreated receptors was much lower than control (Vmax relative to control set at 1 equaled 0.27 ± 0.04, n = 3). However, neither value could be accurately determined for GRK2-treated receptors because of signal-to-noise factors described below, although in any individual experiment the rate of guanine nucleotide exchange in the presence of the highest concentration of Gαq tested was approximately one-fourth that of control receptors (0.26 ± 0.04, n = 3).

Two experiments were performed to evaluate brain βγ saturation with GRK2-treated receptors versus control with a constant concentration of 400 nM Gαq and 1.4 nM receptor (data not shown). The average Kd for control membranes was 297 nM (similar to the experiments performed in Fig. 5 and detailed in the text below) but a Vmax and Kd for GRK2-treated receptors was again difficult to estimate. In individual experiments, the Vmax was 0.34 relative to control.

The difficulty in determining precise values for Kd and Vmax for both Gαq and brain βγ saturation curves was due to the low activity of these receptors relative to control and the low signal to noise ratio. As shown in Fig. 4, the rate of [35S]GTPγS binding to Gαq alone (without added receptor-containing membrane) is as much as 20% of the rate in the presence of treated receptors. Therefore, to determine the saturation curves more precisely and thereby minimize the error in the determinations of the Kd and Vmax, experiments were subsequently performed in which receptor concentration in the assay was adjusted such that control and treated receptors had approximately equal catalytic activity (Fig. 5). For control receptors, the coupling assay was linear between 0.2 and 2 nM (data not shown), so we chose to decrease the level of control receptors in the assay.

Representative saturation curves are shown for Gαq (Fig. 5B) and brain βγ (Fig. 5A). Using a constant concentration of 600 nM brain βγ (twice that used in Fig. 4), the measured Kd for Gαq (mean ± S.D.) was similar among the control (88.3 ± 13.8 nM, n = 4), GRK2-treated (116 ± 35.7 nM, n = 5), and agonist-pretreated (162 ± 54.6 nM, n = 3) GRP-Rs, but there was a large decrease in the Vmax for the GRK2- and agonist-treated receptors relative to control. Vmax values for the treated receptors (relative to control GRP-R Vmax set at 1) were 0.18 ± 0.02 (n = 5) for GRK2-treated, and 0.27 ± 0.02 (n = 3) for agonist-pretreated GRP-Rs. As predicted from the data in Figs. 3 and 4, four to five times as many receptors were needed in the assay to develop reaction velocities similar to controls, and the results for agonist-pretreated GRP-Rs were consistent with those determined using equal concentrations of receptors. A small (less than 2-fold) decrease in affinity (Kd) cannot be ruled out from our data for the agonist-treated receptors. We should note that the measured Kd values for Gαq obtained in the experiments of Fig. 5 are lower than those in the experiments of Fig. 4. This is most likely due to the inclusion of a higher fractional...
Phosphorylation Uncouples the GRP Receptor from \( G_q \)

In this study, we show that the GRP-R is a substrate for \( \text{in vitro} \) agonist-induced phosphorylation by GRK2 with a stoichiometry of \(~1\text{mol PO}_4/\text{mol GRP-R}\). \textit{In vivo} agonist exposure stimulates GRP-R phosphorylation to a similar degree. For both treatments, this stoichiometry of phosphorylation impairs the ability of GRP-R to activate \( G_q \). Uncoupling occurs with two distinct \( G\beta\gamma \) subunit preparations and when the receptor is in two different membrane environments. Furthermore, \textit{in vitro} GRK2 phosphorylation and \textit{in vivo} agonist exposure both reduce the ability of GRP-R to catalyze guanine nucleotide exchange on \( G_q \), with little or no detectable arrestin. Molecular analysis of the uncoupling suggests that the major effect of both treatments is on the \( V_{\text{max}} \) for catalysis of nucleotide exchange rather than the affinity for the receptor-\( G \) protein subunit interactions, implying that the phosphorylated GRP-R can interact with \( G \) protein but is catalytically impaired. Taken together, these data suggest that \textit{in vivo} rapid agonist-induced GRP-R phosphorylation is mediated by a GRK.

We note several differences between the GRP-R and the reported behavior of other GPCRs. First, we find a lower stoichiometry of phosphorylation compared with previous examinations of GPCR phosphorylation (7, 37). We detect only \(~1\) mol \( \text{PO}_4/\text{mol GRP-R} \), which suggests that there may be either one major site of phosphorylation or a few mutually exclusive sites. The \( \beta_2\text{AR} \) and rhodopsin were found to be phosphorylated to different stoichiometries by different GRKs (14, 38), and it has been suggested that only \(~1–2\) mol \( \text{PO}_4/\text{mol receptor} \) may be needed to inactivate rhodopsin (14, 39).

Second, the mechanism of deactivation is different. Whereas the major effect of phosphorylation on rhodopsin activation is on the affinity of rhodopsin for holotransducin (14), our data indicate primarily an effect on \( V_{\text{max}} \). If the stoichiometry is \(<1\) mol \( \text{PO}_4/\text{mol GRP-R} \) and there are phosphorylated and non-phosphorylated GRP-Rs in our assays, our analysis cannot distinguish an effect of phosphorylation on \( V_{\text{max}} \) from a large effect on \( K_m \). But because rhodopsin phosphorylated by GRK2 was coupled (14), although we found that the GRP-R phosphorylated by GRK2 is uncoupled, receptor- and kinase-specific differences may be present.

These data suggest that there may not be a single paradigm for GPCR desensitization. The role of arrestin family members in GPCR desensitization has not been fully clarified, and our data indicate that arrestins may not be needed as universal coupling inhibitors. In fact, Dicker et al. (40) recently published data showing that signaling by the parathyroid hormone receptor is inhibited by the binding of GRKs to the receptor in a phosphorylation-independent fashion, suggesting an alternative mechanism to ours for arrestin-independent uncoupling. This mechanism cannot explain our data, because urea extraction removes all proteins not integral to the membrane, and therefore we should not have GRKs present in Figs. 3–5, in which we see a large effect of phosphorylation on coupling.

In this study, without detectable levels of an arrestin, we document levels of coupling inhibition comparable with those seen in the presence of arrestins for rhodopsin and the \( \beta_2\text{AR} \) (13, 14, 18). Although \( \beta\)-arrestin seems crucial for uncoupling GRK-phosphorylated \( \beta_2\text{AR} \), arrestin may only enhance the arrestin-independent uncoupling of phosphorylated rhodopsin.

Our data suggest that the GRP-R may be biologically more like \( \beta_2\text{AR} \) than the \( \beta_2\text{AR} \) in its desensitization. This seems plausible, because the structure of the GRP-R is more like rhodopsin than the \( \beta_2\text{AR} \) in its desensitization. These structural factors may have functional consequences at the molecular level because the GRK phosphorylation sites on the \( \beta_2\text{AR} \) (38) are not homologous to the RK phosphorylation sites for rhodopsin (39). GRK2 and GRK5 both phosphorylate the

**FIG. 5.** Saturation of the rate of agonist-stimulated guanine nucleotide exchange by \( G_q \) and \( G\beta\gamma \). Comparison of control membranes with GRK2-phosphorylated and \textit{in vivo} agonist-exposed membranes at concentrations generating equal receptor activity. 7 M urea-extracted 5ET4 membranes prepared as described in the legend to Fig. 3 were combined in solution G with 1 \( \mu \)M bombesin, 1 mM Tris, 660 nM GDP, and 8 nM (0.01 \( \mu \text{Ci/\muL} \) [\( ^{35}\text{S}\text{GTP} \)S]. The concentrations of GRP-R in the membranes were as follows: untreated (control) 0.4 nM, GRK2-phosphorylated (BARK-treated) 2 nM, and \textit{in vivo} bombesin exposed (BN-treated) 1.4 nM (A) or 1.6 nM (B). In A, \( G_q \) was fixed at 300 nM and \( G\beta\gamma \) varied from 0–800 nM. Data are presented with a background subtracted from each time point consisting of \( G_a \) incubated under the same conditions without any membrane. In B, \( G\beta\gamma \) was fixed at 600 nM, and \( G_q \) varied from 0–220 nM. Binding reactions proceeded for 15 min at 30 °C, and then binding was determined as described under “Experimental Procedures.” Curves are best-fit using a single site model and are representative of at least three experiments (see text).

saturation of \( \beta\gamma \) in the experiments of Fig. 5. Furthermore, the \( K_{is} \) for \( \beta\gamma \) was also similar among the three treatment conditions when assayed with 300 nM \( G_a \) (control 290 ± 83 nM, \( n = 9 \); GRK2-treated 295 ± 154 nM, \( n = 4 \); and agonist-treated 358 ± 118 nM, \( n = 7 \)), but there was a corresponding decrease in \( V_{\text{max}} \) relative to control (GRK2-treated 0.21 ± 0.02, \( n = 4 \); and agonist-treated 0.27 ± 0.05, \( n = 7 \)). Therefore, the decrease in catalytic activity seen for GRP-Rs either treated with GRK2 \textit{in vitro} or exposed to a saturating concentration of bombesin \textit{in vivo} is mainly due to a decrease in the maximum velocity of catalysis by the GRP-R rather than a decrease in the affinity of the receptor for either the \( \alpha \) or \( \beta\gamma \) subunits.

**DISCUSSION**

In this study we show that the GRP-R is a substrate for \textit{in vitro} agonist-induced phosphorylation by GRK2 with a stoichiometry of ~1mol PO_4/mol GRP-R. In vivo agonist exposure stimulates GRP-R phosphorylation to a similar degree. For both treatments, this stoichiometry of phosphorylation impairs the ability of GRP-R to activate G_q. Uncoupling occurs with two distinct Gβγ subunit preparations and when the receptor is in two different membrane environments. Furthermore, in vitro GRK2 phosphorylation and in vivo agonist exposure both reduce the ability of GRP-R to catalyze guanine nucleotide exchange on G_q with little or no detectable arrestin. Molecular analysis of the uncoupling suggests that the major effect of both treatments is on the V_max for catalysis of nucleotide exchange rather than the affinity for the receptor-G protein subunit interactions, implying that the phosphorylated GRP-R can interact with G protein but is catalytically impaired. Taken together, these data suggest that in vivo rapid agonist-induced GRP-R phosphorylation is mediated by a GRK.

We note several differences between the GRP-R and the reported behavior of other GPCRs. First, we find a lower stoichiometry of phosphorylation compared with previous examinations of GPCR phosphorylation (7, 37). We detect only ~1 mol PO_4/mol GRP-R, which suggests that there may be either one major site of phosphorylation or a few mutually exclusive sites. The β_2AR and rhodopsin were found to be phosphorylated to different stoichiometries by different GRKs (14, 38), and it has been suggested that only 1–2 mol PO_4/mol receptor may be needed to inactivate rhodopsin (14, 39).

Second, the mechanism of deactivation is different. Whereas the major effect of phosphorylation on rhodopsin activation is on the affinity of rhodopsin for holotransducin (14), our data indicate primarily an effect on V_max. If the stoichiometry is <1 mol PO_4/mol GRP-R and there are phosphorylated and non-phosphorylated GRP-Rs in our assays, our analysis cannot distinguish an effect of phosphorylation on V_max from a large effect on K_m. But because rhodopsin phosphorylated by GRK2 was coupled (14), although we found that the GRP-R phosphorylated by GRK2 is uncoupled, receptor- and kinase-specific differences may be present.

These data suggest that there may not be a single paradigm for GPCR desensitization. The role of arrestin family members in GPCR desensitization has not been fully clarified, and our data indicate that arrestins may not be needed as universal coupling inhibitors. In fact, Dicker et al. (40) recently published data showing that signaling by the parathyroid hormone receptor is inhibited by the binding of GRKs to the receptor in a phosphorylation-independent fashion, suggesting an alternative mechanism to ours for arrestin-independent uncoupling. This mechanism cannot explain our data, because urea extraction removes all proteins not integral to the membrane, and therefore we should not have GRKs present in Figs. 3–5, in which we see a large effect of phosphorylation on coupling.

In this study, without detectable levels of an arrestin, we document levels of coupling inhibition comparable with those seen in the presence of arrestins for rhodopsin and the β_2AR (13, 14, 18). Although β-arrestin seems crucial for uncoupling GRK-phosphorylated β_2AR, arrestin may only enhance the arrestin-independent uncoupling of phosphorylated rhodopsin.

Our data suggest that the GRP-R may be biologically more like β_2AR than the β_2AR in its desensitization. This seems plausible, because the structure of the GRP-R is more like rhodopsin than the β_2AR in its desensitization. These structural factors may have functional consequences at the molecular level because the GRK phosphorylation sites on the β_2AR (38) are not homologous to the RK phosphorylation sites for rhodopsin (39). GRK2 and GRK5 both phosphorylate the...
Phosphorylation Uncouples the GRP Receptor from \( G_q \)

\( \beta_AR \) 20–30 residues distal to the homologous sites phosphorylated by RK in rhodopsin. Phosphorylation at these distal sites alone may not have the same ability to impair coupling. Although we do not know the exact site of agonist-induced phosphorylation, mutant receptors with the serines and threonines in the carboxyl-terminal domain of the GRP-R changed to amino acid residues that cannot be phosphorylated are hypophosphorylated \( \text{in vivo} \) after exposure to agonist.\(^2\) This suggests that agonist-induced GRP-R phosphorylation is in the carboxyl-terminal tail and potentially at a location homologous to one of the phosphorylation sites in rhodopsin.

Furthermore, recent studies on the \( \beta_AR \) have suggested additional roles for \( \beta \)-arrestin not universal to all GPCRs. For example, \( \beta \)-arrestin appears to mediate agonist-induced \( \beta_AR \) internalization (42). Rhodopsin does not become internalized, and not all GPCRs that internalize, such as the angiotensin II type 1a receptor, require arrestin for efficient internalization (43). It may be that the angiotensin II type 1a receptor also desensitizes without arrestin. Alternatively, some receptors may require mainly the clathrin recognition function of arrestin for internalization but not the uncoupling function. Additionally, it has been suggested that \( \beta \)-arrestin can also function as an adapter protein to mediate the recruitment of c-Src to the activated \( \beta_AR \) (44). Added complexity in the signal transduction cascade is possible if there are circumstances under which uncoupling of a receptor from heterotrimeric G proteins is independent of \( \beta \)-arrestin association.

Our data do not rule out the possibility that another cofactor is needed to uncouple the GRP-R in the absence of arrestin. If this cofactor is present in our assays, it is most likely a lipid or protein integral to the membrane, because the membrane fraction is not purified beyond the urea extraction and contains uncharacterized proteins and lipids. Alternatively, a factor may be contributed by the \( G_q \) or \( \beta \) that is at sufficient stoichiometry to inhibit coupling but has not been detected by Coomassie Blue staining. There are differences in the site(s) of phosphorylation and mechanisms of desensitization when different GPCRs coupled to \( G_q \) have been analyzed, as well as differences among GPCRs coupled to other G proteins. There are no currently defined consensus sequences indicating sites for receptor phosphorylation \( \text{in vivo} \), which protein kinase(s) are responsible, and what functional consequence(s) result. Our data indicate that GRK-induced phosphorylation of some GPCRs, such as the GRP-R, may desensitize these receptors in the absence of an arrestin homologue. Further work will be needed to determine the role, if any, for an arrestin-like molecule in GRP-R uncoupling, desensitization, internalization, and signaling.

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\(^2\) G. S. Kroog and J. F. Battey, unpublished data.