Mechanism of Quenching of Phototransduction

BINDING COMPETITION BETWEEN ARRESTIN AND TRANSDUCIN FOR PHOSPHORHODOPSIN*

(Received for publication, April 7, 1997)

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Quenching of phototransduction in retinal rod cells involves phosphorylation of photoactivated rhodopsin by the enzyme rhodopsin kinase followed by binding of the protein arrestin. Although it has been proposed that the mechanism of arrestin quenching of visual transduction is via steric exclusion of transducin binding to phosphorylated light-activated rhodopsin (P-Rh*), direct evidence for this mechanism is lacking. In this study, we investigated both the role of rhodopsin phosphorylation in modulating its interaction with arrestin and transducin and the proposed binding competition between arrestin and transducin for P-Rh*. While the β-adrenergic receptor kinase promotes significant arrestin binding to rhodopsin at a phosphorylation stoichiometry of 2 mol/mol, rhodopsin kinase promotes arrestin binding at a stoichiometry of ~0.9 mol/mol. Moreover, while β-adrenergic receptor kinase phosphorylation of rhodopsin only modestly decreases transducin binding and activation, rhodopsin kinase phosphorylation of rhodopsin significantly decreases transducin binding and activation. Finally, arrestin competes effectively with transducin for binding to P-Rh* (50% inhibition at ~1:1 molar ratio of arrestin:transducin) but has no effect on transducin binding to nonphosphorylated light-activated rhodopsin (Rh*), paralleling the functional inhibition by arrestin on P-Rh*-stimulated transducin activation (50% inhibition at ~1:1 molar ratio of arrestin:transducin). These results demonstrate that a major role of rhodopsin phosphorylation is to promote high-affinity arrestin binding and decrease transducin binding thus allowing arrestin to effectively compete with transducin for binding to photoactivated rhodopsin.

Phototransduction mediated by the photoreceptor rhodopsin and hormonal transduction mediated by the β-adrenergic receptor (βAR) serve as excellent model systems for investigations of the molecular events underlying agonist-induced receptor desensitization (reviewed in Refs. 1 and 2). Phosphorylation of light-activated rhodopsin and hormone-activated βAR by the G protein-coupled receptor kinases rhodopsin kinase and β-adrenergic receptor kinase (βARK), respectively, initiates desensitization and leads to partial uncoupling of receptor-G protein interaction. Rapid and complete uncoupling, however, is accomplished by the subsequent binding of the proteins arrestin and β-arrestin, respectively, to the phosphorylated form of the agonist-activated receptor.

Many studies have investigated the effect of receptor phosphorylation on modulating interaction with arrestins. Light-induced binding of visual arrestin to rhodopsin was highly enhanced by rhodopsin phosphorylation (3), and this binding suppressed light-induced cGMP phosphodiesterase activation by rhodopsin (4). Although it has been shown that highly phosphorylated rhodopsin is significantly impaired in its ability to activate transducin, addition of arrestin accelerated the recovery process and further suppressed phosphodiesterase activation, most likely by quenching partially phosphorylated rhodopsin species (4–7). Recently, Puig et al. (8) demonstrated that a heptaphosphopeptide from the C terminus of rhodopsin stimulates arrestin binding to photoactivated forms of rhodopsin, suggesting that at least one function of rhodopsin phosphorylation is to promote high-affinity arrestin interaction with other cytoplasmic regions of rhodopsin. Indeed, the third cytoplasmic domain of rhodopsin appears to be involved in arrestin binding (9). The study by Puig et al. (8) is consistent with the recently proposed model of strict selectivity of arrestin binding to phosphorylated light-activated rhodopsin (10). This model proposes that arrestin interaction with both the phosphorylated rhodopsin C terminus and rhodopsin domains that manifest its activation state results in a conformational change from a low-affinity to a high-affinity binding state in which a secondary binding site becomes accessible for interaction with rhodopsin. The corresponding domain in arrestin for interaction with the phosphorylated C terminus of rhodopsin, the “phosphorylation-recognition” domain, has been localized to a discrete region near the middle of the molecule (11).

In hormonal transduction mediated by the βAR, arrestins have been shown to specifically inhibit the signaling of βARK-phosphorylated β2AR (12–15). Moreover, in vitro synthesized radiolabeled β-arrestin and arrestin 3 were demonstrated to have high-affinity and selectivity for binding to the phosphorylated form of the β2AR and m2 muscarinic acetylcholine receptor (16, 17). Similar to the visual arrestin studies, mutagenesis of β-arrestin and arrestin 3 has also identified regions critical for recognition of the phosphorylated state of the receptor (17).

The effect of receptor phosphorylation on G protein interaction has not been well characterized. Although several studies have demonstrated a modest to potent functional effect of receptor phosphorylation on G protein activation, it is not known whether this effect is due to attenuated G protein binding to

* This work was supported in part by National Institutes of Health Grants GM4944 and GM47419 (to J. L. B.) and Training Grant T32-HL07324 (to J. G. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: βAR, β-adrenergic receptor; βARK, β-adrenergic receptor kinase; GTP-γ-S, guanosine 5′-3-O-(thiotriphosphate); ROS, rod outer segment; DTT, dithiothreitol; AMP-PNP, adenosine 5′-(γ,γ)-methylene triphosphate.

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The receptor or subsequent G protein activation. Phosphorylated rhodopsin was demonstrated to have a significantly lower light-induced phosphodiesterase activating capacity than non-phosphorylated rhodopsin (4, 18), and it was subsequently proposed that rhodopsin phosphorylation reduces the binding affinity for transducin (18). βARK phosphorylation of the βAR resulted in a modest functional inhibition of βAR-stimulated G$_s$-GTPase activity (12, 15), and phosphorylation of the m2 muscarinic acetylcholine receptor by βARK modestly reduced its capacity to stimulate GTPyS binding to G$_s$ (19). The mechanism of arrestin quenching of rhodopsin signaling is believed to involve steric exclusion of transducin interaction with photoactivated rhodopsin (3, 5, 20). In 1984, Kuhn and co-workers (3) demonstrated that excess transducin displaces arrestin from phosphorylated rod outer segment (ROS) membranes suggesting a binding competition between arrestin and transducin for site(s) on the cytoplasmic surface of phototransduced rhodopsin. A quantitative assessment of this competition, however, could not be made due to the crude nature of the protein preparations. Here, we quantitatively characterized the ability of arrestin to compete with transducin for binding to nonphosphorylated and phosphorylated light-activated rhodopsin (Rh$^-$ and P-Rh$^-$), respectively, and correlated these results with the functional effects of arrestin on Rh$^-$ and P-Rh$^-$-stimulated transducin activation.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP was from NEN Life Sciences Products. 11-cis-Retinal was generously provided by Dr. R. K. Crouch (Medical University of South Carolina). Frozen bovine retinas were purchased from George A. Hormel and Co. The bovine arrestin cDNA was generously provided by Dr. T. Shinohara (National Institutes of Health) (21). A monoclonal antibody (F4C1) against a conserved N-terminal domain of all arrestins (22) was kindly provided by Dr. L. Donoso (Wills Eye Hospital). A polyclonal antibody (116) against Gi protein was generously provided by Dr. D. Manning (University of Pennsylvania). All restriction enzymes and most other molecular biology reagents were kindly provided by Dr. D. Lefkowitz (Duke University Medical Center). ROS membranes (23) were prepared as described (9) with all manipulations carried out on ice. Bovine arrestin was isolated from ROS membranes suggesting a binding competition between arrestin and transducin for site(s) on the cytoplasmic surface of photoactivated rhodopsin. A quantitative assessment of this competition, however, could not be made due to the crude nature of the protein preparations. Here, we quantitatively characterized the ability of arrestin to compete with transducin for binding to nonphosphorylated and phosphorylated light-activated rhodopsin (Rh$^-$ and P-Rh$^-$), respectively, and correlated these results with the functional effects of arrestin on Rh$^-$ and P-Rh$^-$-stimulated transducin activation.

ROS Membrane Preparation—Urea-treated ROS membranes were prepared as described (9) with all manipulations carried out on ice under dim red light. Briefly, 50 mg frozen bovine retinas were resuspended in 50 ml of 10 mM Tris acetate, pH 7.4, 65 mM NaCl, 2 mM MgCl$_2$, 34% (w/v) sucrose, shaken vigorously and centrifuged at 2000 $g$ for 5 min. The supernatant was diluted with 2 volumes of 10 mM Tris acetate, pH 7.4, and centrifuged as above. The crude ROS pellet was resuspended in 30 ml of 10 mM Tris acetate, pH 7.4, 1 mM MgCl$_2$, 0.77 $m$ sucrose and further purified on a discontinuous sucrose gradient. The interface between 0.84 and 1.0 $m$ sucrose was collected, diluted 2-fold with 10 ml 10 mM Tris acetate, pH 7.4, and centrifuged at 48,000 $g$ for 20 min. The resulting pellet was resuspended in 50 ml of 10 mM Tris acetate, pH 8.0, 5 mM EDTA, 5 mM urea, sonicated on ice for 4 min, diluted with 2 volumes of 50 mM Tris-HCl, pH 7.4, and centrifuged at 100,000 $g$ for 45 min. The pellet was washed three times with 50 ml Tris-HCl, pH 7.4, resuspended in the same buffer, sonicated on ice, snap frozen in liquid nitrogen, and stored in the dark at ~80 °C. The resulting supernatant was assayed for absorbance at 498 nm using an extinction coefficient of 40,600. Purity of the ROS membrane preparation was assessed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining.

Phosphorylation of Rhodopsin—ROS membranes were phosphorylated by βARK or rhodopsin kinase. βARK was purified from Sf9 insect cells infected with a recombinant baculovirus as described previously (24). Rhodopsin kinase was purified to ~50% homogeneity by sequential Q-Sepharose and heparin-Sepharose chromatography. Briefly, the Sf9 supernatant (20 ml, 30 mg) was diluted 2-fold with 2 mM Tris-HCl, pH 7.4, 0.5 mM phenylmethanesulfonyl fluoride, 20 µg/ml leupeptin, 200 µg/ml benzamidine (Buffer A). The column was washed with 0.25–0.5 ml with an 5-ml Sepharose column equilibrated with Buffer A. The column was washed with Buffer A and arrestin eluted at 1 ml/min with a 120-ml linear gradient of NaCl (400 mM) in Buffer A. Peak fractions (between 65 and 150 mM NaCl) were pooled (30 ml, 2–3 $g$), concentrated to 200 ml and boiled in 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 20 µg/ml leupeptin, 200 µg/ml benzamidine and then loaded at 0.5 ml/min on a 5-ml Q-Sepharose column equilibrated with Buffer A. The column was washed with 200 ml NaCl in Buffer A and arrestin eluted at 0.5 ml/min with a 40-ml linear gradient of NaCl (200–600 mM) in Buffer A. Peak fractions (between 370 and 470 mM NaCl) were pooled (10 ml, 100 µg), concentrated to 0.25–0.5 µg/ml with an Amicon concentrator, and aliquots were snap frozen in liquid nitrogen and stored at ~80 °C. Arrestin levels and purity were assessed by immunoblotting, using the monoclonal antibody F4C1, and with Coomassie Blue staining of 10% SDS-polyacrylamide gels.

Transducin Purification from Bovine Retina—Isolation of holotransducin from dark-adapted bovine retinas was performed as described (25). Briefly, ROS membranes were bleached in white light for 30 min, pelleted, and resuspended in hypotonic buffer (10 mM Hepes, pH 7.5, 1 mM DTT, 0.1 mM EDTA, 0.3 mM phenylmethanesulfonyl fluoride), repeating the latter manipulation several times. Transducin was then recovered by resuspending the membranes in hypotonic buffer containing 100 µM GTP, incubation on ice for 30 min under constant illumination, and centrifugation. This step was repeated three times and the supernatants pooled, concentrated, aliquoted, and stored at ~80 °C. Purity was assessed by Coomassie Blue staining of a 10% SDS-polyacrylamide gel.

Centrifugation Binding Assay and Western Analysis—Arrestin and transducin alone or together were incubated with rhodopsin or phosphorhodopsin for 5 min at 30 °C under constant illumination in 10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 0.75 mM MgCl$_2$, 0.2 mM DTT, 1 mg/ml...
bovine serum albumin in a total volume of 50 μl. Samples were then placed on ice and loaded, under dim red light, onto 0.2 ml of the above buffer containing 0.2 M sucrose. Following centrifugation at 100,000 rpm for 30 min at 4 °C, supernatants were removed and the resulting pellets solubilized in 20 μl of SDS sample buffer, electrophoresed on a 10% SDS-polyacrylamide gel, and electroblotted onto nitrocellulose for 1 h at 100 V. Transferred proteins were then analyzed using the F4C1 monoclonal arrestin antibody and/or the 116 polyclonal G$_i$ antibody. The 116 polyclonal G$_i$ antibody is directed against a C-terminal region of G$_i$, and selectively detects G$_i$1 and G$_i$2 to a lesser extent G$_i$3 and G$_i$4 (23). Blots were blocked for 30 min with 5% (w/v) nonfat dry milk in Buffer B (20 mM Tris- HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), incubated with a 1:5000 (F4C1) and/or 1:500 (116) dilution of primary antibody in Buffer B with milk for 1 h, washed with Buffer B, incubated for 1 h with a 1:2000 dilution of anti-mouse and/or anti-rabbit secondary antibody (for F4C1 and 116, respectively) in Buffer B with milk, washed with Buffer B, and then visualized using ECL reagent (Amersham) followed by autoradiography. Films were scanned with a densitometer (Molecular Dynamics) and the intensity of immunoreactive bands in standard samples loaded on the same gel.

Nonspecific binding was determined in the absence of rhodopsin and endogenous immunoreactivity determined in the presence of rhodopsin alone.

**GTPase Assay**—Transducin alone or in the presence of increasing concentrations of arrestin was incubated with rhodopsin or phosphorhodopsin for 5 or 15 min at 30 °C under constant illumination in 10 mM endogenous immunoreactivity determined in the presence of rhodopsin. Nonspecific binding was determined in the absence of rhodopsin and immunoreactive bands in standard samples loaded on the same gel. Nonspecific binding was determined in the absence of rhodopsin and endogenous immunoreactivity determined in the presence of rhodopsin alone.

Since direct evidence for competition between arrestins and G proteins for receptor binding is lacking, we assessed the ability of purified preparations of arrestin and transducin to bind to rhodopsin using a centrifugation binding assay (to isolate rhodopsin-bound proteins). Transducin was purified from bovine retinas using a standard procedure in which transducin is allowed to bind to photoactivated rhodopsin, peripheral proteins are then removed by hypotonic buffer washes and transducin subsequently released by GTP. This protein preparation was estimated to be ≥95% pure (Fig. 1, lane 2). Arrestin was overexpressed in Sf9 insect cells using the baculovirus expression system and purified by sequential Q-Sepharose and heparin-Sepharose chromatography. This protein preparation was judged to be ≥75% pure (Fig. 1, lane 2). The ROS membrane preparation contained ≥90% rhodopsin (Fig. 1, lane 3), and Western analysis indicated negligible endogenous arrestin and transducin (data not shown). Since the expression and purification of arrestin in Sf9 insect cells has not previously been described, we initially investigated the function of this protein. Purified Sf9-expressed arrestin was found to inhibit in vitro synthesized [3H]arrestin binding to P-Rh with 50% inhibition occurring at an ~1.6 mol/mol of arrestin added to each sample. Each curve is the mean ± S.E. of three or four independent experiments (for P-Rh) or the mean ± S.D. of two independent experiments (for Rh). Nonspecific sedimentation of arrestin was ~7% of the total input.

**RESULTS AND DISCUSSION**

Since direct evidence for competition between arrestins and G proteins for receptor binding is lacking, we assessed the ability of purified preparations of arrestin and transducin to bind to rhodopsin using a centrifugation binding assay (to isolate rhodopsin-bound proteins). Transducin was purified from bovine retinas using a standard procedure in which transducin is allowed to bind to photoactivated rhodopsin, peripheral proteins are then removed by hypotonic buffer washes and transducin subsequently released by GTP. This protein preparation was estimated to be ≥95% pure (Fig. 1, lane 2). Arrestin was overexpressed in Sf9 insect cells using the baculovirus expression system and purified by sequential Q-Sepharose and heparin-Sepharose chromatography. This protein preparation was judged to be ≥75% pure (Fig. 1, lane 2). The ROS membrane preparation contained ≥90% rhodopsin (Fig. 1, lane 3), and Western analysis indicated negligible endogenous arrestin and transducin (data not shown). Since the expression and purification of arrestin in Sf9 insect cells has not previously been described, we initially investigated the function of this protein. Purified Sf9-expressed arrestin was found to inhibit in vitro synthesized [3H]arrestin binding to P-Rh with 50% inhibition occurring at an ~1.6 mol/mol of arrestin added to each sample. Each curve is the mean ± S.E. of three or four independent experiments (for P-Rh) or the mean ± S.D. of two independent experiments (for Rh). Nonspecific sedimentation of arrestin was ~7% of the total input.

**Fig. 1. Polyacrylamide gel analysis of protein preparations.** ~1–2 μg of each protein preparation was electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were visualized by Coomassie Blue staining. Lane 1, transducin; lane 2, arrestin; lane 3, urea-treated rod outer segments.

**Fig. 2. Arrestin binding to rhodopsin and phosphorhodopsin.** Sf9-expressed arrestin (25 nM) was incubated with either Rh+ or βARK (A)- or RK (B)-phosphorylated P-Rh+ (0–200 nM) for 5 min at 30 °C in a total volume of 50 μl in the light. Samples were then processed as described under “Experimental Procedures” and analyzed by Western analysis using the F4C1 arrestin monoclonal antibody. Following visualization of blots with ECL, autoradiograms were scanned with a densitometer (Molecular Dynamics) and the intensity of immunoreactive bands in experimental samples compared with the intensity of immunoreactive bands in standard samples loaded on the same gel. Endogenous arrestin immunoreactivity in the ROS membrane preparation was negligible. Data are expressed as the amount of arrestin bound to rhodopsin relative to the total amount of arrestin added to each sample. Each curve is the mean ± S.E. of three or four independent experiments (for P-Rh+) or the mean ± S.D. of two independent experiments (for Rh+). Nonspecific sedimentation of arrestin was ~7% of the total input.
It was recently demonstrated that βARK phosphorylation of rhodopsin to a stoichiometry of 2 mol/mol is necessary and sufficient for high-affinity [3H]arrestin binding to rhodopsin (10). The arrestin binding differences apparent in Fig. 2A may be explained in light of this study and the observation that a phosphorhodopsin preparation, containing a specific average phosphorylation stoichiometry, is actually a heterogeneous population of rhodopsin molecules where $67\%$ of the total molecules contain the average phosphorylation stoichiometry or greater (29). Thus, the modestly enhanced binding of arrestin to P-Rh* ($4.6$ mol/mol) is likely due to a greater percentage of rhodopsin molecules in the P-Rh* ($2.7$ mol/mol) preparation containing less than 2 mol/mol stoichiometry. Similarly, the significantly decreased binding of arrestin to P-Rh* ($1.6$ mol/mol) when compared with P-Rh* ($2.7$ mol/mol) is likely due to a significantly greater fraction of rhodopsin molecules in the P-Rh* ($1.6$ mol/mol) preparation containing a phosphorylation stoichiometry of less than 2 mol/mol.

Interestingly, arrestin binding to rhodopsin phosphorylated by rhodopsin kinase (RK) to $0.9$ mol/mol was almost as good as its binding to P-Rh* phosphorylated by RK to 2–3 mol/mol stoichiometries (Fig. 2B). Moreover, arrestin binding to P-Rh* phosphorylated by RK to $0.9$ mol/mol was in fact better than its binding to P-Rh* phosphorylated by βARK to $1.6$ mol/mol. Thus, high-affinity binding of arrestin to RK-phosphorylated rhodopsin appears to require incorporation of only 1 mol of phosphate/molecule of rhodopsin. These observations clearly indicate that RK and βARK differ in their initial site of phosphorylation of rhodopsin. Indeed, a recent study (30) demonstrating that the initial C-terminal site phosphorylated by RK and βARK is different, with RK phosphorylating Ser338 first and Ser343 second while βARK has the reverse preference, sheds light on this observed difference in arrestin binding. A plausible explanation is that phosphorylation of Ser338 is necessary and sufficient for high-affinity arrestin binding. Consistent with the apparent requirement of $1$ mol/mol phosphorylation stoichiometry for high-affinity arrestin binding to RK-phosphorylated rhodopsin, several studies have indicated that one phosphate incorporated into rhodopsin is sufficient for arrestin function. In a functional assay, total rhodopsin inactivation is
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Fig. 4. A, arrestin/transducin binding competition for phosphorylated rhodopsin. Transducin (50 nM) was incubated with Rh or βARK-phosphorylated P-Rh* (−4.6 mol/mol) (50 nM) in the absence or presence of increasing concentrations of arrestin (0–200 nM) for 5 min at 30 °C in the light in a total volume of 50 μL. Samples were then processed as described under “Experimental Procedures” and analyzed by Western analysis using both the arrestin and G_{α} antibodies. This gel is representative of three independent experiments each with similar results. B, competition between arrestin and transducin for binding to Rh* and P-Rh*. Following visualization of blots with ECL, autoradiograms were scanned by a densitometer and the intensity of immunoreactive transducin bands in experimental samples compared with standards loaded on the same gel. Data are expressed as the percent of transducin interaction. Transducin (50 nM) was incubated with Rh* or P-Rh* (50 nM) in the absence or presence of increasing concentrations of arrestin (0–200 nM) in the presence of [γ-32P]GTP for 15 min at 30 °C in the light in a total volume of 50 μL. Reactions were quenched as described under “Experimental Procedures” and femtomole of GTP hydrolyzed determined by scintillation counting of aliquots of the supernatants following centrifugation. Data are expressed as the percent of rhodopsin-stimulated GTPase activity in the absence of added arrestin. Control GTPase activity with P-Rh* and Rh* was ~10 and ~17 fmol/min, respectively. Curves are mean ± S.E. of three independent experiments (for P-Rh*) and mean ± S.D. of two independent experiments (for Rh*).

The finding that phosphorylation of Ser^{338} is likely a key modulator of arrestin binding, along with our recent identification of Arg^{175} as a key phosphorylation-sensitive trigger in arrestin (11, 31), implies that phosphoserine 338 in rhodopsin may directly interact with Arg^{175} in arrestin. Moreover, several phosphorylation-independent arrestin mutants bound equally well to P-Rh* and 329G-Rh* (truncated rhodopsin lacking the C-terminal phosphorylation sites), but weakly to Rh*. This suggests that rhodopsin phosphorylation induces a conformational rearrangement involving the C terminus that promotes high-affinity arrestin binding.

Since the direct binding of transducin to rhodopsin and phosphorhodopsin has not been well characterized, we next investigated the effect of rhodopsin phosphorylation on this interaction. Similar to the arrestin binding studies, we compared transducin interaction with rhodopsin phosphorylated by either βARK or RK. Phosphorylation of rhodopsin by βARK to −4.6 mol/mol modestly reduced transducin binding (Fig. 3A), correlating with the modest decrease in the ability of βARK-phosphorylated rhodopsin (−4.6 mol/mol) to stimulate the GTPase activity of transducin (Fig. 3B). Interestingly, similar to the more potent effects of rhodopsin phosphorylation by RK on arrestin binding, phosphorylation by RK also more effectively decreased transducin interaction. Rhodopsin kinase phosphorylation of rhodopsin to −0.9 mol/mol significantly decreased transducin binding (Fig. 3C), correlating with a significant reduction in RK-phosphorylated P-Rh*–stimulated transducin GTPase activity (Fig. 3D). The more potent effect on transducin interaction of rhodopsin phosphorylation by RK compared with βARK is highlighted by the observation that the decrease in transducin binding and activation is more pronounced with the RK-phosphorylated P-Rh* than the βARK-phosphorylated P-Rh*. The significant decrease in transducin activation by RK phosphorylation of rhodopsin is consistent with the desensitization observed in arrestin-knockout mice (32).

This is the first study to date to correlate the reduced ability of the phosphorylated receptor to both bind and activate the G protein. Although it has been proposed that phosphorylation of rhodopsin reduces its binding affinity for transducin (18), evidence to support this is lacking. Multiple studies have demonstrated that the rhodopsin domains most critical for transducin binding are the second and third cytoplasmic loops (33–36). Moreover, one study has suggested involvement of the C terminus of rhodopsin in transducin interaction (37), although truncation mutagenesis suggested that the C terminus is not absolutely required for transducin interaction (38). Possible explanations for reduced transducin binding to phosphorhodopsin are that phosphorylation of the C terminus may directly decrease transducin interaction with the C terminus or it may result in a conformational change that alters the accessibility of other cytoplasmic regions of rhodopsin critical for transducin interaction.

The more potent effects on transducin interaction by RK compared with βARK phosphorylation of rhodopsin again suggest mechanistic differences in the manner in which these G protein-coupled receptor kinases phosphorylate rhodopsin. In light of the study indicating that RK initially phosphorylates Ser^{338} (30), these results strongly suggest that phosphorylation of Ser^{338} is a key modulator of both transducin and arrestin interaction. Since βARK phosphorylation of rhodopsin to a high
stoichiometry only modestly decreased transducin interaction, the sequential order of phosphorylation of rhodopsin may be more critical for modulating transducin interaction than phosphorylation of Ser<sup>338</sup> per se. It is conceivable that initial phosphorylation of Ser<sup>338</sup> is required for a specific conformational change to occur that significantly decreases transducin interaction. Thus, monophosphorhodopsin phosphorylated on Ser<sup>338</sup> may exist in a conformation particularly unfavorable for transducin interaction, a conformation that is subsequently maintained throughout further phosphorylation of rhodopsin by RK. However, in the case of βARK phosphorylation of rhodopsin, this unique change may not occur since Ser<sup>343</sup> is phosphorylated before Ser<sup>338</sup>.

Using the purified preparations of arrestin and transducin, we next investigated whether these proteins compete for binding to rhodopsin. Since βARK-phosphorylated rhodopsin demonstrates both high-affinity binding to arrestin and a modest reduction in transducin interaction, we assessed the binding competition with this form of phosphorylated rhodopsin. Increasing concentrations of arrestin were incubated with transducin and either Rh<sup>-</sup> or P-Rh<sup>*</sup> (−4.6 mol/mol), and rhodopsin-bound arrestin and transducin were then isolated by centrifugation through a 0.2 m sucrose cushion. The proteins were resolved by electrophoresis and binding quantitated by immunoblotting using arrestin and Gi<sub>α</sub> antibodies. It can be clearly observed that as arrestin binding to P-Rh<sup>*</sup> increased, transducin binding decreased (Fig. 4A). Arrestin competed with transducin for binding to P-Rh<sup>*</sup> with 50% inhibition occurring at a molar ratio of arrestin:transducin of ~1:1 (−50 nM arrestin) and maximal inhibition of ~80% at the highest concentration of arrestin (Fig. 4B). Arrestin, however, did not compete with transducin for binding to Rh<sup>-</sup> (Fig. 4B), consistent with its low affinity for nonphosphorylated rhodopsin (Fig. 2A and Refs. 10 and 27).

To our knowledge, this represents the first time that an arrestin protein has been shown to directly inhibit G protein binding to the phosphorylated form of an agonist-activated receptor, thus providing direct evidence for the binding competition mechanism first proposed by Kuhn et al. (3). Similar to the demonstration that excess transducin displaces arrestin from binding to highly phosphorylated ROS membranes (3), these results suggest that arrestins and G proteins compete for binding to a common site(s) on the phosphorylated receptor. Based on our previous results that the third cytoplasmic domain of rhodopsin is critical for rhodopsin-arrestin interaction (9), at least one of these overlapping binding site(s) is likely to be the third cytoplasmic loop of rhodopsin.

To assess whether the observed binding competition between arrestin and transducin for phosphorhodopsin can fully explain the functional effect of arrestin on phosphorhodopsin-stimulated transducin GTPase activity. Arrestin inhibited P-Rh<sup>*</sup>-stimulated transducin GTPase activity with 50% inhibition occurring at a molar ratio of arrestin:transducin of ~1:7:1 (−85 nM arrestin) and maximal inhibition of ~70% at the highest concentration of arrestin (Fig. 4C), comparable to the half-maximal value and maximal inhibition observed in the binding competition experiments. Arrestin, however, had no effect on Rh<sup>-</sup>-stimulated transducin GTPase activity (Fig. 4C), consistent with its inability to compete with transducin for binding to this form of rhodopsin (Fig. 4B). The molar ratio of arrestin:P-Rh<sup>*</sup> required for 50% inhibition in the functional studies here correlates well with the molar ratio of arrestin:receptor required for 50% inhibition in functional studies reported elsewhere (12–14, 39). These results thus indicate that the observed binding competition between arrestin and transducin for interaction with phosphorylated rhodopsin is able to explain the functional effects of arrestin on inhibiting phosphorhodopsin-stimulated transducin activation.

In summary, we investigated the proposed mechanism of action of arrestins (involving a binding competition with G proteins for the phosphorylated agonist-activated receptor) and correlated these results with the functional effects of arrestins on receptor-stimulated G protein activation. We also characterized the direct effect of receptor phosphorylation on interaction with both arrestins and G proteins. Our results demonstrate: 1) phosphorylation of rhodopsin greatly enhances arrestin binding and reduces transducin binding and activation; 2) RK is more effective than βARK at phosphorylating rhodopsin and modulating its interaction with arrestin and transducin; and 3) arrestin competes with transducin for binding to P-Rh<sup>*</sup> paralleling the functional inhibition of arrestin on P-Rh<sup>*</sup>-stimulated transducin activation. Finally, the data presented here raise the intriguing possibility that phosphorylation of GPRs to different stoichiometries with various G protein-coupled receptor kinases, and then assessment of the effect on GPR interaction with arrestins and G proteins, may be a viable approach for investigation of the specificity of GPR interaction with G protein-coupled receptor kinases and arrestins.

Acknowledgments—We thank Deidre Heyser for purified transducin and Drs. Jon Erickson and Dr. Richard Cerione for helpful discussions.

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