Role of Receptor-attached Phosphates in Binding of Visual and Non-visual Arrestins to G Protein-coupled Receptors*5

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Background: The relative contribution of phosphates and active GPCR conformation is unknown.

Results: Using WT and mutant arrestins and receptors, we show that phosphates are critical for arrestin binding to some GPCRs but not to others.

Conclusion: The role of receptor-attached phosphates in arrestin binding varies widely depending on the arrestin-receptor combination.

Significance: Distinct molecular mechanisms mediate arrestin recruitment to different GPCRs.

Arrestins are a small family of proteins that regulate G protein-coupled receptors (GPCRs). Arrestins specifically bind to phosphorylated active receptors, terminating G protein coupling, targeting receptors to endocytic vesicles, and initiating G protein-independent signaling. The interaction of rhodopsin-phosphorylated active receptors, terminating G protein-coupled receptors (GPCRs). Arrestins specifically bind to greater in the case of phosphorylation, its role in the recruitment of arrestin-2 and -3 is much lower compared to arrestin binding varies depending on the receptor-arrestin interaction. Here we tested the role of conserved lysines in homologous positions of non-visual arrestins by generating K2A mutants in which both lysines were replaced with alanines. K2A mutations in arrestin-1, -2, and -3 significantly reduced their binding to active phosphorylhosin in vitro. The interaction of arrestins with several GPCRs in intact cells was monitored by a bioluminescence resonance energy transfer (BRET)-based assay. BRET data confirmed the role of Lys-14 and Lys-15 in arrestin-1 binding to non-cognate receptors. However, this was not the case for non-visual arrestins in which the K2A mutations had little effect on net BRETmax values for the M2 muscarinic acetylcholine (M2R), β2-adrenergic (β2AR), or D2 dopamine receptors. Moreover, a phosphorylation-deficient mutant of M2R interacted with wild type non-visual arrestins normally, whereas phosphorylation-deficient β2AR mutants bound arrestins at 20–50% of the level of wild type β2AR. Thus, the contribution of receptor-attached phosphates to arrestin binding varies depending on the receptor-arrestin pair. Although arrestin-1 always depends on receptor phosphorylation, its role in the recruitment of arrestin-2 and -3 is much greater in the case of β2AR than M2R and D2 dopamine receptor.

G protein-coupled receptors (GPCRs); also known as seven-transmembrane domain receptors) are encoded by ~800 genes in humans and up to ~3,400 in other mammals (SEVENs database). GPCRs are the largest family of signaling proteins targeted by more than 40% of clinically used drugs, although less than 10% of human GPCRs are actually targeted (1). The GPCR superfamily is extremely diverse with less than 20% sequence identity shared by all members (1, 2). Attenuation of G protein signaling is as essential to GPCR function as receptor activation. Agonist-activated GPCRs become substrates of G protein-coupled receptor kinases (GRKs) (3, 4), which phosphorylate serine and threonine residues in the receptor C terminus and/or intracellular loops (3, 5). Arrestin binding to active phoshoceptors sterically blocks G protein-protein interactions (6, 7), effectively quenching G protein-mediated signaling (3). Receptor-bound arrestins interact with numerous non-receptor partners, initiating G protein-independent signaling (8–10) and linking GPCRs to the endocytic machinery (11, 12), thereby initiating internalization (13).

In contrast to the large GPCR family, in mammals arrestins are represented by four subtypes (14). Arrestin-1 and arrestin-4 are restricted to photoreceptors where they regulate structurally similar rhodopsin and cone opsins (15). In contrast, ubiquitously expressed arrestin-2 and -3 (5) apparently interact with the widely varied binding interfaces offered by other GPCRs. Crystal structures of arrestin-1 (16), -2 (17), -3 (18), and -4 (19) reveal a highly conserved characteristic two-domain fold shared by very few other proteins (20). In the basal state, the C-tail folds back onto the N-domain, forming a three-element interaction with β-strand I and α-helix I mediated by hydrophobic interactions.

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1 This article contains supplemental Figs. S1 and S2.

2 The abbreviations used are: GPCR, G protein-coupled receptor; BRET, bioluminescence resonance energy transfer; M2R, M2 muscarinic acetylcholine receptor; β2AR, β2-adrenergic receptor; D2R, D2 dopamine receptor; GRK, G protein-coupled receptor kinase; P-Rh*, light-activated phosphorhodopsin; RLuc8, Renilla luciferase variant 8; IRES, internal ribosome entry sequence; F/L, fluorescence/luminescence.

3 We use systematic names of arrestin proteins: arrestin-1 (historic names, S-antigen, 48-kDa protein, and visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons, its gene is called “arrestin 3” in the HUGO database).
phobic side chains (21). In addition, a conserved arginine in the C-tail constitutes part of the main arrestin phosphate sensor, the polar core (16, 22).

Several lines of evidence suggest that receptor binding is accompanied by a global conformational rearrangement of arrestin (23–26). Based on the crystal structure (16) and extensive mutagenesis (22, 27–31), a model of arrestin activation was proposed (for reviews, see Refs. 15 and 32). First, phosphorylated residues on rhodopsin bind two lysines on β-strand I, Lys-14 and Lys-15 in bovine arrestin-1, or homologous non-visual arrestin residues (see Fig. 1, A and B) (14), forcing the shift of their side chains. This change in β-strand I disrupts the three-element interaction and allows the lysines to deliver the phosphates to buried Arg-175 in the polar core (21). The receptor-attached phosphates neutralize the charge on Arg-175, disrupting its interaction with Asp-296 and destabilizing the polar core (22). Breakup of the three-element interaction also releases the unstructured arrestin C-tail (25, 26, 33), removing Arg-382 from the polar core and further destabilizing it. The disruption of these constraining intramolecular interactions allows the arrestin transition into an active receptor-binding conformation (32).

The role of Lys-14 and Lys-15 in arrestin-1 as primary phosphate sensors is well established (21). However, this mechanism was assumed to operate in non-visual arrestins essentially by extension (5, 34). To test the role of homologous lysines in the arrestin transition into an active receptor-binding conformation (32).

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**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP, [14C]leucine, and [3H]leucine were from PerkinElmer Life Sciences. All restriction and DNA-modifying enzymes (T4 DNA ligase, Vent® DNA polymerase, and calf intestine alkaline phosphatase) were from New England Biolabs (Ipswich, MA). Rabbit reticulocyte lysate was from Ambion (Austin, TX). SP6 RNA polymerase was prepared as described (35). Cell culture reagents and media were from Mediatech (Manassas, VA) or Invitrogen. The luciferase substrate coelenterazine- h was from Discoverx (Fremont, CA). All other reagents were from Amresco (Solon, OH) or Sigma-Aldrich.

**Mutagenesis and Plasmid Construction**—Bovine arrestin-1 cDNA (36) was a gift from Dr. T. Shinohara (National Eye Institute, National Institutes of Health, Bethesda, MD). Plasmids that encode bovine arrestin-1, the long splice variant of bovine arrestin-2 (37, 38), and the short splice variant of arrestin-3 (37, 39) with engineered unique restriction sites were described previously (40, 41). The K2A, NCA, and KNC mutations (a comprehensive list of mutated amino acids is provided in Fig. 1C) were introduced by PCR using an appropriate mutagenizing oligonucleotide as a forward primer and an oligonucleotide downstream from the far restriction site to be used for subcloning as a reverse primer. Resulting fragments of various lengths and an appropriate primer upstream of the near restriction site were then used as reverse and forward primers, respectively, for the second round of PCR. The resulting fragments were purified, digested with the corresponding restriction enzymes, and subcloned into the appropriately digested pGEM-2 in vitro transcription plasmids (Promega, Madison, WI) encoding respective arrestins. All constructs were confirmed by dideoxy sequencing.

Arrestins N-terminally tagged with Venus (a variant of enhanced yellow fluorescent protein (42); a gift from Dr. J. A. Javitch, Columbia University, New York, NY) were engineered using pGEM2-based constructs. Venus was amplified by PCR using a forward primer that adds EcoRI and AsISi sites upstream of the start codon and a reverse primer that codes for a short spacer with the “SGLKSRRALDS” sequence and an in-frame NcI site as described previously (18, 43). Venus was subcloned between the EcoRI and NcI restriction sites. The arrestins were subcloned in-frame with the Venus-spacer sequence using NcI and HindIII sites. The Venus-arrestin fusion proteins were subcloned into a pcDNA3 mammalian expression vector (Invitrogen), which was modified as described (44, 45), using the EcoRI and HindIII restriction sites.

A plasmid encoding *Renilla* luciferase variant 8 (RLuc8) (46) was a gift from Dr. Nevin A. Lambert (Medical College of Georgia, Augusta, GA). RLuc8 was fused in-frame with the sequence of the triple HA-tagged human M2R, β2AR, and D2R from the cDNA Resource Center. A modified pcDNA3-based backbone was prepared by replacing the multiple cloning site with the EcoRI-AsISi-SbfI-Ascl-Xhol-PacI-HindIII restriction sites. These sites were used to clone the coding sequences of all GPCRs, which were amplified by PCR using forward primers that introduce EcoRI and AsISi restriction sites upstream of the receptor start codon and reverse primers that introduce an in-frame SbfI site. These fragments were subcloned using AsISi and SbfI in-frame with C-terminal RLuc8 flanked by the in-frame SbfI and Ascl.

The resulting P3HM2Luc plasmid was used as the base to introduce the ST8A mutations that eliminate the putative GRK phosphorylation sites on the third intracellular loop of M2R (S286A, T287A, S288A, S290A, T307A, S309A, T310A, and S311A) as described (47, 48). Two previously described GRK phosphorylation-deficient mutants of the β2AR (termed here S3A and ST15GA) provided by Drs. Richard B. Clark (University of Texas Health Science Center, Houston, TX) and Jeffrey L. Benovic (Thomas Jefferson University, Philadelphia, PA), respectively, were used as templates to introduce these mutations into the β2AR-RLuc8-bearing plasmid P3HB2ALuc. ST15GA includes S261A, S262A, S345A, S346A, S355A, S356G, T360A, S364G, T384A, T393A, S396G, S401A, S407G,
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T408A, and S411A (49, 50), whereas S3A includes S355A, S356A, and S364A (51–53). Mutant β2-AR coding sequences were amplified by PCR using a forward primer that adds KpnI and a reverse primer that adds SbfI restriction sites.

Bicistronic versions of the receptor-RLuc8 pcDNA3 based plasmids were made for the co-expression of human GRK2. For this, an internal ribosome entry sequence (IRES) was amplified from pIRE2-GFP (Clontech) with appropriate forward and reverse primers that add Ascl and XhoI sites, respectively. The pBluescript-based plasmid with the coding sequence of GRK2 was from Dr. Antonio De Blasi (Istituto di Ricerche Farmacologiche Mario Negri, Santa Maria Imbaro, Italy). The GRK2 sequence was subcloned into the pcDNA3-receptor-RLuc8-IRES constructs by PCR amplification using forward and reverse primers adding XhoI and PacI-HindIII sites, respectively.

In Vitro Transcription, Translation, Rhodopsin Preparation, and P-Rh* Binding Assay—In vitro transcription, translation, rhodopsin preparation, and P-Rh* binding assay were performed as described recently (43).

GRK2 Quantification by Western Blot—COS-7 cells were transfected with the indicated plasmids as described for BRET assays, exactly replicating the amounts of receptor-RLuc8- and GRK2-bearing constructs, and Venus-arrestins or Venus alone. 48 h post-transfection, cells were lysed with lysis buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Nonidet P-40, protease inhibitor mixture from Roche Applied Science) on ice for 10 min. Cell debris was pelleted by centrifugation for 10 min at 10,000 × g. Soluble proteins were precipitated with 9 volumes of methanol, pelleted by centrifugation, washed with 90% methanol, air-dried, and dissolved in Laemmli SDS sample buffer at 1 mg/ml. The proteins were subjected to SDS-PAGE (10% gel) and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). GRK2 was visualized using rabbit anti-GRK2 antibody (C-15; Santa Cruz Biotechnology, sc-562; 1:500) followed by anti-rabbit horseradish peroxidase-conjugated secondary antibodies from Jackson Immunoresearch Laboratories (West Grove, PA). Protein bands were visualized by enhanced chemiluminescence (ECL; Pierce) and exposed to x-ray film. Optical densities were quantified using a VersaDoc gel imaging system and analyzed with Quantity One software version 4.6.9 (Bio-Rad). The statistical significance of the differences was determined by a Student’s t test or one-way analysis of variance with Dunnett’s multiple comparison test where appropriate using Prism 6.0. Co-immunoprecipitation data were analyzed by one-way analysis of variance with arrestin-receptor combination as the main factor followed by a Bonferroni/Dunn post hoc test with correction for multiple comparisons.

RESULTS

Role of β-Strand I Lysines in P-Rh* Binding of Non-visual Arrestins—According to the current model of arrestin-GPCR interaction, arrestin transition into a high affinity receptor-binding state is triggered by simultaneous engagement of phosphate-binding arrestin residues by receptor-attached phosphates and additional arrestin elements by the parts of the receptor that change conformation upon activation (32). Phosphate-binding Lys-14 and Lys-15 were shown previously to be required for high affinity WT arrestin-1 binding to P-Rh* (21). The positive charges in these positions are conserved in arrestin elements by the parts of the receptor that change conformation upon activation (32). Phosphate-binding arrestin residues by receptor-attached phosphates and additional arrestin elements by the parts of the receptor that change conformation upon activation (32). Phosphate-binding Lys-14 and Lys-15 were shown previously to be required for high affinity WT arrestin-1 binding to P-Rh* (21). The positive charges in these positions are conserved in arrestin elements by the parts of the receptor that change conformation upon activation (32).
homologous residues in arrestin-2 and arrestin-3 (Lys-10,11 and Lys-11,12, respectively) (Fig. 1), we mutated these lysines to alanines in all three subtypes and compared the ability of the resulting K2A mutants to bind P-Rh* in vitro (Fig. 2). The results confirmed previous reports that all three arrestins bind P-Rh* albeit at different levels (57). In all cases, the binding of K2A mutants was reduced 6–7-fold relative to the parental WT protein (Fig. 2), demonstrating that the two lysines in β-strand I play a key role in the interaction of all arrestin subtypes with P-Rh*.

**Role of N-terminal Phosphate-binding Element of Visual Arrestin-1 in Its Interactions with Non-cognate Receptors**—Arrestin-1 was shown previously to be recruited to non-cognate GPCRs expressed in cells (58). Therefore, we tested the functional role of Lys-14 and Lys-15 on β-strand I in its binding to non-visual receptors using BRET between luciferase-tagged receptors and Venus-tagged arrestin-1 (Fig. 3). Arrestin-1 con-
tains an additional phosphate-binding residue absent in other subtypes (19) and demonstrates the most dramatic phosphorylation-dependent increase in receptor binding in the arrestin family (57, 59). As could be expected, WT arrestin-1 better associated with M2R or β2AR upon overexpression of GRK2 in COS-7 cells that have a low level of endogenous GRKs (Fig. 3 and supplemental Fig. S1). Using bicistronic receptor-RLuc8-IRES-GRK2 constructs, we increased the expression of GRK2 2-fold (Fig. 3G). These data suggest that significant arrestin-1 binding to non-visual GPCRs requires high receptor phosphorylation. Interestingly, for the D2R-arrestin-1 pair, net BRET values significantly different from zero were not obtained even upon GRK2 overexpression in COS-7 (Fig. 3, C and F) or HEK-293 cells (not shown), suggesting that arrestin-1 does not appreciably bind D2R regardless of receptor phosphorylation.

WT arrestin-1 demonstrates saturable agonist-induced binding to M2R and β2AR, measured as the difference between the BRET ratio in the presence and absence of agonist (net BRET) (Fig. 3, D and E), in agreement with our previous finding that arrestin-1 binds purified active phosphorylated M2R and β2AR with nanomolar affinity (57). The K2A mutation significantly reduces the BRET ratio observed in the absence of agonist (Fig. 3, A and B) and causes an even more dramatic decrease in net BRET (Fig. 3, D and E). We recently found that the elim-
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Elimination of 10 non-phosphate-binding residues on the concave sides of the two arrestin domains (NCA mutation; Fig. 1C) abolishes the binding of arrestin-1 to non-cognate receptors (43). The combination of NCA and K2A mutations (KNC mutation; Fig. 1C) further suppresses the BRET ratio (Fig. 3, A and B) and completely eliminates the agonist-induced increase in BRET (Fig. 3, D and E). These data suggest that the BRET ratio observed with KNC likely reflects nonspecific “bystander” BRET. Indeed, co-expression of similar levels of Venus with these receptors yields an even higher BRET ratio than observed with the KNC mutant, likely due to the much smaller size and consequent faster diffusion of free Venus than the Venus-arrestin fusion (supplemental Fig. S2). The dependence of the BRET ratio on the Venus expression level with all three receptors looks similar and is not affected by agonists. M2R curves are slightly right-shifted relative to the β2AR and D2R curves, suggesting that luciferase on M2R is somewhat less accessible to freely diffusing cytoplasmic proteins, such as Venus (supplemental Fig. S2). The fact that both WT and K2A arrestin-1 yield the same significantly higher BRET level than the NCA and KNC mutants suggests that there is basal receptor binding of arrestin-1 that is eliminated by the 10 and 12 alanine substitutions in NCA and KNC, respectively. Two lines of evidence indicate that agonists enhance the interaction via increased GRK phosphorylation of the receptor. First, it is not observed without GRK overexpression (Fig. 3 and supplemental Fig. S1). Second, the elimination of phosphate-binding lysines by the K2A mutation virtually abolishes the agonist-dependent increase in binding. Interestingly, with both receptors, about half of the BRET signal exceeding NCA/KNC levels reflects basal interaction, whereas the other half is agonist-dependent. The difference in the ability of the NCA and KNC mutants to bind M2R and β2AR is only marginal, suggesting that in the absence of arrestin-1 interactions with the non-phosphorylated receptor elements (precluded by the NCA mutation (43)) phosphate binding alone cannot support the formation of the arrestin-1 complex with these GPCRs (Fig. 3).

Role of Phosphate-binding Lysines in β-Strand I of Non-visual Arrestins in Receptor Interaction—In contrast to arrestin-1 binding to non-cognate receptors (Fig. 3), the association of non-visual arrestins with the M2R and β2AR does not require GRK2 overexpression (Figs. 4 and 5).

The most striking difference between visual and non-visual arrestin binding to M2R, β2AR, and D2R is that K2A mutations in arrestin-2 and -3 have essentially no effect on the observed BRET ratio in the absence of agonist or on the net BRET increase after agonist stimulation (Figs. 4 and 5). In the case of the binding of arrestin-2 to M2R, both the BRET ratio and net BRETmax are even significantly enhanced by the K2A mutation as compared with WT arrestin-2 (Fig. 4, A and B). Thus, it appears that receptor-attached phosphates play a much less important role in the formation of complexes of both non-visual arrestins with M2R, β2AR, and D2R (Figs. 4 and 5) than in their binding to P-Rh* (Fig. 2).

NCA mutations eliminate 10 non-phosphate-binding residues (Fig. 1) that determine the receptor specificity of arrestin proteins (43) likely by engaging GPCR elements that change conformation upon activation. In contrast to K2A, the effects of the NCA mutations are profound and receptor-dependent. The NCA mutations significantly reduce both basal arrestin-2 and -3 interactions with non-stimulated receptors (reflected by the BRET ratio in the absence of agonists) and virtually eliminate the agonist-induced increase in binding (Figs. 4 and 5). The elimination of the two lysines (K2A) in addition to the NCA mutations, yielding the KNC mutants, only marginally reduces the binding (reflected by net BRETmax) relative to NCA alone (Figs. 4 and 5).

Interestingly, the NCA mutations in arrestins are not as detrimental to their binding to β2AR: the mutants demonstrate considerable basal (agonist-independent) binding and retain the ability to respond to agonist activation (Figs. 4, C and D, and 5, C and D). The elimination of the two phosphate-binding lysines on the NCA background (yielding the KNC mutants) does not significantly change their interactions with β2AR as compared with NCA (Figs. 4, C and D, and 5, C and D). In fact, the net BRETmax of the NCA and KNC mutants of arrestin-2 was 52 and 41% of the WT protein and a comparable 38 and 23% for the same mutants on arrestin-3, respectively (Figs. 4, C and D, and 5, C and D). These data suggest that although the 10 residues eliminated by the NCA mutations play a decisive role in the arrestin-2 and -3 interactions with M2R and D2R additional elements of both non-visual arrestins mediate a significant proportion of their binding to β2AR.

Elimination of Receptor Phosphorylation Sites in β2AR and M2R Differentially Affects Binding of Non-visual Arrestins—Alanine substitution of the two key phosphate-binding lysines in β-strand I by the K2A mutations virtually eliminates the ability of non-visual arrestins to bind P-Rh* (Fig. 2) and the ability of arrestin-1 to interact with M2R and β2AR (Fig. 3) in full agreement with the accepted model of arrestin activation by receptor-attached phosphates (32). The unexpected finding that homologous K2A mutations in arrestin-2 and -3 do not significantly reduce their interactions with M2R, β2AR, and D2R (Figs. 4 and 5) appears to contradict this model. The role of receptor-attached phosphates in arrestin recruitment can also be tested by the elimination of the relevant phosphorylation sites in GPCRs where these serines and threonines were identified. Therefore, next we used previously characterized phosphorylation-deficient mutants of M2R (47, 48) and β2AR (49–53).

We generated the constructs of these mutants C-terminally fused to RLuc8, similar to the corresponding WT receptors. To this end, we used the phosphorylation-deficient M2R-ST8A in which four serine/threonine residues in each of the two clusters in the third cytoplasmic loop of the receptor that were shown to be phosphorylated by GRKs (47, 48) were mutated to alanines. In agreement with the observation that the K2A mutants of both non-visual arrestins bind M2R like WT proteins (Figs. 4 and 5), we found that M2R-ST8A interacts with arrestin-2 and -3 at least as well as WT M2R (Fig. 6, A–D). The elimination of eight phosphorylation sites did not change the BRET ratio or agonist-induced increase in the arrestin-receptor interaction. If anything, it seemed to increase apparent affinity as all BRET curves were left-shifted, indicating that the same level of interaction was achieved at lower arrestin expression. Thus, both arrestin (Figs. 4 and 5) and receptor (Fig. 6) mutagenesis indi-
cates that receptor-attached phosphates do not play a decisive role in arrestin-2 and -3 binding to M2R.

Two phosphorylation-deficient forms of the β2AR have been described. In one, the β2AR-ST15GA mutant, all 15 C-terminal serines and threonines were mutated to alanines or glycines, ablating all possible phosphorylation sites (49, 50). In the other mutant, β2AR-S3A, only a limited set of mutations (S355A, S356A, and S364A) was introduced, which was reported to similarly abolish GRK phosphorylation, arrestin recruitment, receptor desensitization, and internalization (51, 50, 61). The phosphorylation of these sites in the proximal C terminus of β2AR in cells was independently confirmed by mass spectrometry (62). We compared both of these mutants with WT β2AR (Fig. 6, E–H). In contrast to M2R, the elimination of β2AR phosphorylation sites significantly affected arrestin binding. Agonist-induced increase in the arrestin-receptor BRET was reduced by ~50 and ~75% by the S3A and ST15GA mutations, respectively, although the difference between the two mutants was not statistically significant (Fig. 6, E–H). Thus, the phosphorylation of β2AR, particularly at Ser-355, Ser-356, and Ser-364, significantly contributes to the binding of non-visual arrestins.

The striking receptor-dependent difference of the role of phosphates in arrestin binding was rather unexpected. Therefore, we tested our findings by an independent method, co-immunoprecipitation of WT and K2A forms of arrestin-3 with WT and phosphorylation-deficient mutants of M2R and β2AR (Fig. 7). To this end, WT arrestin-3 was co-expressed with WT receptors, M2R-ST8A, and β2AR-ST15GA, whereas arrestin-3-K2A was co-expressed with both WT receptors. Receptors were immunoprecipitated via an N-terminal HA tag, and the amount of receptors and Venus-arrestin-3 in the immunoprecipitate was determined by Western blot (Fig. 7). In agreement with the BRET data (Fig. 5), we did not detect significant differences in the amount of WT and K2A arrestin-3 immunoprecipitating with agonist-activated WT M2R or β2AR (Fig. 7, B)

FIGURE 4. Arrestin-2 recruitment to M2R, β2AR, and D2R does not require phosphate-binding lysines in β-strand I. A–F, BRET-based assays to assess the interaction of increasing amounts of WT (circles) or K2A (squares), NCA (inverted triangles), and KNC (triangles) mutant forms of Venus-arrestin-2 (Arr-2) with luciferase-tagged human M2R (A and B), β2AR (C and D), or D2R (E and F). A, C, and E, BRET ratio in the presence of agonist (Ag) (filled symbols, solid lines) or vehicle (open symbols, dashed lines) in COS-7 cells co-expressing varying amounts of Venus-arrestin-2 with the indicated RLuc8-tagged receptors. Vertical bracketed lines on the right show the agonist-induced increase in BRET. B, D, and F, net BRET (agonist-induced increase in BRET) as a function of Venus-arrestin-2 expression normalized by RLuc8 luminescence (F/L). The symbols are the same as in A, C, and E. Means ± S.E of six repeats in a representative experiment (of three performed) are shown: *, p < 0.05; **, p < 0.01; ****, p < 0.0001.
and C). These data confirm that the elimination of the two phosphate-binding lysines in arrestin-3 does not significantly affect its interactions with M2R and β2AR. Agonist-occupied M2R-ST8A tends to yield a higher BRET ratio with both arrestin-2 and -3 than did WT M2R (Fig. 6, A and C), whereas the agonist-induced increase in binding remains unaffected by the ST8A mutation in the receptor (Fig. 6, B and D). In agreement with these data, we detected a significantly higher level of co-immunoprecipitation of arrestin-3 with M2R-ST8A than with WT M2R (Fig. 7 B). This result suggests that co-immunoprecipitation likely reflects the sum of the agonist-independent binding and agonist-induced increase in the interaction, suggesting that in the case of M2R both have high enough affinity to survive the procedure. Importantly, in the same type of experiment, β2AR-ST15GA demonstrated a significantly lower ability to co-immunoprecipitate arrestin-3 than did WT β2AR (Fig. 7C). This results correlates well with the greatly reduced agonist-dependent recruitment of arrestin-3 to the β2AR-}

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ST15GA mutant (Fig. 6H) rather than with its agonist-independent interaction with Venus-arrestin-3 reflected by the BRET ratio (Fig. 6G). Thus, it appears that in the case of β2AR mostly agonist-induced binding survives the co-immunoprecipitation procedure. Thus, the comparison of BRET data, which reflect essentially the equilibrium between arrestin-bound and free receptor, and co-immunoprecipitation, which detects only relatively high affinity interactions that survive extensive washes, suggests that the strength of arrestin “predocking” to non-stimulated receptor varies with different GPCRs.

**DISCUSSION**

Crystal structures of all four vertebrate arrestins in their basal conformation (16–19) are remarkably similar: an elongated molecule with two cuplike domains held together by several intramolecular interactions highly conserved in evolution (14). High Arrhenius activation energy (23), dramatic changes in the accessibility of the C-tail to proteases upon binding to the
receptor and polyanionic heparin that mimics phosphoreceptor (24, 31), the increase of the mobility of C-terminal residues (33), and direct distance measurements between the arrestin C-tail and the N-domain (25, 26) suggested that receptor binding is accompanied by a global conformational rearrangement in arrestin that includes the release of the C-tail. This conformational change is believed to be the hallmark of arrestin transition into an active high affinity receptor-binding state.

The prevailing mechanistic model of arrestin activation by GPCRs posits that it requires simultaneous engagement of the

**FIGURE 6. Binding of non-visual arrestins to WT and phosphorylation-deficient M2R and β2AR.** A–H, BRET-based assays to assess the interaction of increasing amounts of indicated Venus-tagged arrestins (Arr) with luciferase-tagged WT or phosphorylation-deficient (ST8A) M2R (A–D) and WT β2AR and its mutants lacking 15 (ST15GA) or three (S3A) GRK phosphorylation sites (E–H). A, C, E, and G, BRET ratio in the presence of agonist (Ag) (filled symbols, solid lines) or vehicle (open symbols, dashed lines) in COS-7 cells co-expressing varying amounts of Venus-tagged arrestins with the indicated RLuc8-tagged receptors. Vertical bracketed lines on the right show the agonist-induced increase in BRET. B, D, F, and H, net BRET (agonist-induced increase in BRET) as a function of the expression of the indicated Venus-tagged arrestins normalized by RLuc8 luminescence (F/L). The symbols are the same as in A, C, E, and G. Means ± S.E. of six repeats in a representative experiment (of two performed) are shown: **, p < 0.01; ***, p < 0.001.
arrestin “phosphate sensor” by receptor-attached phosphates and the “activation sensor” by parts of the receptor that change conformation upon agonist activation (for reviews, see Refs. 15 and 32). The activation sensor was tentatively localized to the interdomain interface (63), but its exact mechanism of action remains unclear (5). In contrast, the molecular mechanism of arrestin activation by receptor-attached phosphates is fairly well established. The “polar core,” an unusual arrangement of five solvent-excluded charged residues between the two arrestin domains (16), was identified as the key phosphate sensor.

The breakdown of the salt bridge between an arginine and aspartic acid in the polar core by charge reversal mutations yielded phosphorylation-independent forms of arrestin-1 (22, 29, 30, 64) and other subtypes (19, 65–67) that bind active forms of their cognate receptor regardless of their phosphorylation. Two highly exposed lysines in β-strand I (Fig. 1) bind phosphates first and guide them to the polar core (21). Their alanine substitution in arrestin-1 was shown to suppress the binding to P-Rh* of WT arrestin-1 with an intact polar core but not of the mutants with destabilized basal conformation (21). The conservation of these lysines in all arrestins from C. elegans to mammals (14) suggested that receptor-attached phosphates likely play a similar role in the activation of all arrestin subtypes.

Our recent finding that alanine substitution of 10 non-phosphate-binding residues that determine receptor preference of arrestin proteins virtually obliterates the binding of arrestin-2 and -3 to several GPCRs in living cells (43) prompted us to rigorously test this assumption experimentally with different arrestin-receptor combinations. We used several complementary approaches to evaluate the role of receptor-attached phosphates in arrestin binding. First, we compared the binding of WT arrestin-1, -2, and -3 and corresponding K2A mutants in which the two lysines were substituted with alanines to P-Rh* in vitro (Fig. 2). We found that this mutation reduces the binding of all three subtypes to active phosphorhodopsin ~6–7-fold in agreement with the idea that receptor-attached phosphates are indispensable for arrestin activation by P-Rh*. Moreover, the K2A mutation in arrestin-1 virtually blocked the agonist-induced increase in arrestin binding to M2R and β₂AR in cells (Fig. 3), suggesting that the phosphates on these two receptors are equally important for the recruitment of arrestin-1.

However, arrestin-2 and -3 binding to M2R, β₂AR, and D2R in the cellular context brought a surprising result: K2A mutations in both non-visual arrestins had very little effect on these interactions (Figs. 4 and 5). The fact that the NCA mutations in these experiments yielded a profound reduction in receptor binding showed that the interaction is highly sensitive to the changes of the receptor-binding surface of arrestin (Figs. 4 and 5). Two mechanisms can account for these results: either receptor-attached phosphates play a much lesser role in the interaction of non-visual arrestins with these receptors or the phosphate-binding residues remaining in the K2A mutants, such as previously identified positive charges in the loop between β-strands IX and X and on β-strand X (29, 63), can successfully guide the phosphates to the polar core of non-visual arrestins in the absence of the lysines in β-strand I. To discriminate between these possibilities, we used previously characterized phosphorylation-deficient mutants of M2R (47, 48) and β₂AR (49–53) (Fig. 6). The results of these experiments suggested that the answer for these two GPCR subtypes is different. In the case of M2R, neither elimination of the two key phosphate-binding lysines in arrestin (Figs. 4, 5, and 7) nor elimination of key GRK targets on the receptor (Figs. 6 and 7) significantly reduces arrestin binding. Thus, the phosphates attached by GRKs to the third cytoplasmic loop of M2R do not play a decisive role in arrestin recruitment and activation. In contrast, the elimination of phosphorylation sites in β₂AR dramatically reduced arrestin binding (Figs. 6 and 7), suggesting that the lack
of effect of K2A mutations (Figs. 4 and 5) is largely due to the ability of other phosphate-binding residues in the two non-visual arrestins to take over their functional role. Importantly, both NCA and KNC mutants of arrestin-2 and -3 retain a significant proportion of their ability to bind WT β2AR in an agonist-dependent manner (Figs. 4 and 5), suggesting that this interaction can be mediated by the phosphates binding to remaining positive charges in the K2A mutants and possibly additional receptor-binding arrestin residues that are not engaged by the M2R or D2R.

Collectively, our data show that the relative role of phosphates and other receptor elements in the recruitment and activation of arrestins is distinct for different arrestin-receptor combinations. In the case of P-Rhβ binding, both receptor-attached phosphates and the two lysines in β-strand I of all arrestins are indispensable (Fig. 2). This is also true for arrestin-1 interactions with M2R and β2AR (Fig. 3), suggesting that this is likely a general rule for arrestin-1 binding to any GPCR. The binding of arrestin-2 and -3 to the M2R is at the opposite end of the spectrum: the elimination of the two lysines in arrestins (Figs. 4, 5, and 7) or phosphorylation sites in the receptor (Figs. 6 and 7) does not appreciably affect the binding, whereas the elimination of 10 non-phosphate-binding arrestin residues completely abolishes it (Figs. 4 and 5). Because the results with arrestin-2 and -3 are virtually identical, these data indicate that M2R elements other than phosphates largely drive arrestin binding. It is worth noting that these results do not exclude the possibility that negatively charged residues in M2R interacting with the positive charges that remained intact in the K2A mutants activate arrestins in lieu of phosphates. This would not be unprecedented: the role in arrestin binding of intrinsic negative charges in the luteinizing hormone/choriogonadotropin receptor (68), leukotriene B4 receptors (69), and chemokine decay receptor D6 (70) has been demonstrated. The binding of arrestin-2 and -3 to β2AR is in between these extreme cases: both NCA mutations (Figs. 4 and 5) and elimination of the phosphorylation sites (Figs. 6 and 7) reduce but do not completely block the interaction, implicating receptor-attached phosphates and other receptor elements, as predicted by the accepted model (5) and supported by experimental evidence obtained with this receptor (71). Because the addition of the K2A mutation to NCA (yielding the KNC mutants) does not reduce the binding to a greater extent than NCA alone, phosphates must be interacting with other positive charges on the arrestin surface.

Accumulating evidence suggests that arrestins can form functionally different complexes with the same receptor depending on the number of receptor-attached phosphates (72) and the positions where receptors are phosphorylated (73), which appears to be determined by the GRK that attaches the phosphates (74, 75). Where receptor-attached phosphates play a major role in arrestin recruitment, their positions apparently determine functional consequences of the arrestin binding to the phosphoreceptor (76–78). In the context of our data, it is tempting to speculate that one of the mechanisms underlying the formation of distinct arrestin-receptor complexes may be the engagement of the lysines on β-strand I or alternative phosphate-binding positive charges by differentially localized receptor-attached phosphates.

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