Conservation of the Phosphate-sensitive Elements in the Arrestin Family of Proteins*

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Arrestins play a key role in the homologous desensitization of G protein-coupled receptors (GPCRs). These cytosolic proteins selectively bind to the agonist-activated and GPCR kinase-phosphorylated forms of the GPCR, precluding its further interaction with the G protein. Certain mutations in visual arrestin yield “constitutively active” proteins that bind with high affinity to the light-activated form of rhodopsin without requiring phosphorylation. The crystal structure of visual arrestin shows that these activating mutations perturb two groups of intramolecular interactions that keep arrestin in its basal (inactive) state. Here we introduced homologous mutations into arrestin2 and arrestin3 and found that the resulting mutants bind to the β2-adrenoceptor in vitro in a phosphorylation-independent fashion. The same mutants effectively desensitize both the β2-adrenergic and δ-opioid receptors in the absence of receptor phosphorylation in Xenopus oocytes. Moreover, the arrestin mutants also desensitize the truncated δ-opioid receptor from which the C terminus, containing critical phosphorylation sites, has been removed. Conservation of the phosphate-sensitive hot spots in non-visual arrestins suggests that the overall fold is similar to that of visual arrestin and that the mechanisms whereby receptor-attached phosphates drive arrestin transition into the active binding competent state are conserved throughout the arrestin family of proteins.

Signaling by various members of the superfamily of G protein-coupled receptors (GPCRs) is attenuated by a uniform two-step mechanism (1). First, the agonist-activated receptor that catalyzes GDP/GTP exchange on G proteins is specifically phosphorylated by a G protein-coupled receptor kinase (GRK). An arrestin protein then binds to the active phosphoreceptor, which makes further G protein interaction impossible by simple steric exclusion (2). Because of the high affinity of non-visual arrestins2 for various components of the trafficking machinery (3–5), the arrestin-receptor complex is then internalized. Arrestin binding also serves to switch GPCR signaling from G proteins to various mitogen-activated protein kinases (6–8). The loss of active receptor conformation in the endosomes (presumably due to ligand dissociation) facilitates arrestin dissociation, rendering the phosphoreceptor accessible to protein phosphatases. The dephosphorylated receptor can then be recycled back to the plasma membrane (9).

Perfectly timed binding and dissociation of arrestins, which are ensured by their remarkable selectivity for the phosphorylated/activated form of their cognate receptors, are equally important for high fidelity of this quenching mechanism. Destabilization of certain intramolecular interactions that support the basal (inactive) arrestin conformation by the activated phosphoreceptor is the mechanistic basis of arrestin selectivity (10, 15, 19, 20). The visual arrestin residues involved in the stabilization of its basal state have been identified by extensive mutagenesis (10–15, 24, 26) and crystallography (19). Here we describe homologous mutations in both non-visual arrestins that also yield proteins with constitutive activity, suggesting that the basal conformation of all arrestins and the activating mechanisms triggering arrestin transition into its high affinity receptor binding state are conserved throughout this family of proteins. The results presented in this study help to define the conserved mechanisms of arrestin activation.

**EXPERIMENTAL PROCEDURES**

Materials—[γ-32P]ATP, [14C]leucine, and [3H]leucine were purchased from PerkinElmer Life Sciences. All restriction enzymes were purchased from New England Biolabs. Sepharose 2B and all other chemicals were from the sources previously described (13, 16). Rabbit reticulocyte lysate and SP6 RNA polymerase were prepared as previously described (20, 21). 11-cis-retinal was generously supplied by Dr. R. K. Crouch.

Site-directed Mutagenesis—Arrestin3 (22) and arrestin2 (16) cDNAs were subcloned into the pG2S6-I plasmid (10) to yield plasmids pARR3 and pBARR, respectively. These pGEM2-based plasmids encoding bovine wild-type arrestins with an idealized 5′-untranslated region (20) under control of the SP6 promoter were used for all further mutagenesis. All mutations 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 appropriate restriction endonucleases, and subcloned into appropriately digested pARR3 or pBARR. The sequence of all constructs was confirmed by dideoxy sequencing.

In Vitro Transcription, Translation, and Evaluation of Mutant Stability—Plasmids were linearized with HindIII before in vitro transcrip-

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§ The abbreviations used are: GPCR, G protein-coupled receptor; GRK, GPCR kinase; β2AR, β2-adrenergic receptor; P-β2AR, phosphorylated β2AR; P-β2AR*, phosphorylated isoproterenol-activated β2AR; β2AR*, isoproterenol-activated β2AR; β2AR, antagonist-occupied β2AR; P-β2AR*, antagonist-occupied phosphorylated β2AR; 3A, triple alanine substitution (F375A, V376A, F377A) in the C-tail of arrestin and 1286A, V375A, F377A, F388A in C-tails of arrestin2 and 3; WT, wild type; Rh, unphosphorylated dark rhodopsin; P-Rh, dark phosphorylated rhodopsin; Rh*, light-activated, unphosphorylated rhodopsin; P-Rh*, light-activated phosphorylated rhodopsin; DOR, δ-opioid receptor.

Note that here we use the systematic names of non-visual arrestins. The synonyms of arrestin2 are β-arrestin and β-arrestin1; arrestin3 is also called β-arrestin2.
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...tion to produce mRNAs encoding full-length arrestin proteins. In vitro transcription and translation in rabbit reticulocyte lysate were performed as previously described (13, 16, 20, 21). All arrestin proteins were labeled by incorporation of [3H]leucine and [14C]leucine with the specific activity of the mix, 1.5–3 Ci/mmol, resulting in specific activity of arrestin proteins within the range of 54–90 Ci/mmol (120–200 dpm/mmol). Translation of each of the arrestin mutants used in this study produced a single labeled protein band with the expected mobility on SDS-PAGE. Two parameters were used for assessment of mutant stability (14). First, protein yields in the in vitro translation are known to correlate with stability because misfolded or denatured proteins are rapidly destroyed by proteases present in rabbit reticulocyte lysate (14). Second, the purified proteins tend to aggregate and are pelleted by centrifugation at 350,000 × g for 1 h. As an estimate of a mutant’s relative stability, we used its yield multiplied by the percentage of protein remaining in the supernatant after incubation for 10 min at 37°C followed by centrifugation. This integral parameter calculated for a mutant was expressed as a percent of that for wild-type arrestin (14). The relative stability of all mutants used in this study exceeds 80%.

Receptor Preparations—Urea-treated rod outer segment membranes were prepared, phosphorylated with rhodopsin kinase, and regenerated with 11-cis-retinal as described (10). A purified, reconstituted β2-adrenergic receptor (β2AR) was prepared and phosphorylated with a β-adrenergic receptor kinase (GRK2) as described (13–15). The stoichiometry of phosphorylation for the rhodopsin and β2AR preparations used in these studies was 2.7–3.8 mol of phosphate/mol of receptor.

Direct Binding Assay—A direct binding assay was performed as described (13, 16–18). In vitro translated tritiated arrestins (50 fmol) were mixed in 50 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, 50 mM potassium acetate with phosphorylated or unphosphorylated β2AR (100 fmol/assay) activated by 100 µM isoproterenol, or with 7.5 pmol of various functional forms of rhodopsin in a final volume of 50 µL. The samples were then incubated for 35 min at 30°C (β2AR) or 5 min at 37°C (rhodopsin), immediately cooled on ice, and loaded onto 2-mL Sepharose 2B columns equilibrated with 10 mM Tris-HCl, pH 7.5, and 100 mM NaCl. Bound arrestin eluted with the receptor-containing membranes in the void volume (between 0.5 and 1.1 ml). Non-specific binding determined in the presence of 0.3 µg of liposomes (<10% of the total binding and <1% of the arrestin present in the assay) was subtracted.

Desensitization Studies in Xenopus Oocytes—Stage IV oocytes from Xenopus laevis frogs were harvested, defolliculated, and cultured as described (18). cRNAs were prepared from cDNA templates using the Message Machine kit (Ambion, Austin, TX). cDNAs (GenBank™ accession numbers in parentheses) for rat GRK3 (AA144588), human β2AR (AI052644), mouse β-tyrosine receptor (DOR) (L60322), and rat G protein-gated inwardly rectifying potassium channel subunits Kir3.1 (U01071) and Kir3.4 (X83384) were amplified and linearized prior to cRNA synthesis. cDNAs for all forms of bovine arrestin2 (M33601) and arrestin3 (L14641) were first amplified by PCR using oligonucleotides designed to add the T7 promoter upstream and a 45-base poly(A) tail downstream of the respective open reading frames. Standard two-electrode voltage clamp recordings were performed to register Kir3 currents activated by agonist perfusion, as described (26).

The expression levels of all forms of arrestin in oocytes were determined by quantitative Western blotting with F4C1 monoclonal anti-arrestin antibody (27) using the corresponding purified arrestins as standards as described (12). Arrestin expression levels were between 1.6 and 4 pmol/µg of total protein.

RESULTS

Two main groups of intramolecular interactions are largely responsible for the stability of the basal conformation of visual arrestin (14, 15, 19, 25). One is an unusual network of five residues involved in bulk hydrophobic residues in either par-...
that homologous mutations in all arrestins yield similar phenotypes. As predicted, arrestin3 (1–393), which does not have the distant C-tail but has all the key elements of both the polar core and the three-element interaction, shows essentially WT selectivity for P-β2AR*

Interestingly, the most potent phosphorylation-independent form of visual arrestin (Fig. 2B), arrestin3(1A), that binds with very high affinity to unphosphorylated, light-activated rhodopsin (Rh*) (Fig. 2B), does not demonstrate enhanced binding to β2AR*, although its binding to P-β2AR* is about 50% higher than that of WT visual arrestin (Fig. 2A). Similarly, constitutively active forms of both non-visual arrestins demonstrate higher binding to light-activated phosphorhodopsin (P-Rh*) than their parental WT proteins, but none of these mutants shows an enhanced binding to Rh* (Fig. 2B). Thus, mutational destabilization of either of the two main intramolecular interactions that hold arrestin proteins in the basal state facilitates the binding of these proteins to the phosphorylated active form of a non-preferred receptor but is not sufficient to promote binding to its unphosphorylated active form. The constitutively active forms of arrestin2 and arrestin3 that bind equally well to the phosphorylated and unphosphorylated forms of β2AR* still strongly prefer the phosphorylated form of rhodopsin. To further explore the mechanism underlying constitutive activity of the mutants, we also tested the binding of the most potent phosphorylation-independent forms (3A mutants) to the inactive forms of both receptors. In the case of β2AR, the latter were represented by both empty (P-β2AR and β2AR*) and antagonist-occupied (in the presence of 10 μM alprenolol, P-β2ARα and β2ARα, forms (Fig. 3A), whereas in the case of rhodopsin a dark inactive receptor was used (P-Rh and Rh) (Fig. 3B). As we reported earlier (16), the binding of visual arrestin to any form of β2AR is low and appears to depend only on receptor phosphorylation (i.e. the binding to phosphorylated forms is higher than to unphosphorylated forms and it does not depend on receptor activation state). 3A mutation enhances the binding without changing this pattern. Both WT non-visual arrestins demonstrate relatively high binding to inactive forms of both phosphoreceptors. 3A mutations enhance their binding to all phosphorylated forms, further reducing their ability to discriminate between active and inactive phosphoreceptor (Fig. 3A). However, the binding of these mutations to an unphosphorylated receptor is strictly activation-dependent; both 3A mutants bind to β2AR* with high affinity and do not demonstrate appreciable binding to β2AR or β2ARα (Fig. 3A). In contrast to the strong preference for activated β2AR*, the binding of constitutively active non-visual arrestins to rhodopsin was not significantly enhanced by receptor activation (compare Rh and Rh* binding on Fig. 3B).

Next we compared the ability of these arrestins to promote desensitization of β2AR in Xenopus oocytes with and without simultaneous expression of GRK3. The major advantage of this system is that in sharp contrast to cultured cells oocytes do not express detectable amounts of arrestins or GRKs. As a result, the direct effects of mutant arrestins and GRKs on the rate of receptor desensitization in a living cell can be tested without any interference from endogenous proteins. Another major advantage is an easy readout; activation of a Gααq or Gαq protein by any GPCR can be monitored in real time via a potassium current through a βγ-gated inwardly rectifying channel (18, 26). As shown in Fig. 4, the expression of WT arrestins does not accelerate the rate of β2AR desensitization, whereas the coexpression of GRK3 with either arrestin accelerates it 3–4-fold. In contrast, arrestin2 (3A) and several arrestin3 mutants (3A, 1–392, 3AR3Q, and R170E) dramatically accelerate β2AR inactivation even in the absence of GRK3. All phosphorylation-independent mutants fully retain the ability to inactivate GRK3-phosphorylated receptor. This is true even for arrestin3 (R170E) in which the charge of phosphate-binding Arg-170 is reversed, likely precluding direct interaction of this residue with the phosphate. Thus, high affinity arrestin binding to β2AR is possible when its interactions with phosphates is either precluded by their absence on the receptor or impeded by the absence of one of the key phosphate binding arrestin residues. These results suggest that the binding of non-visual arrestins to receptor-attached phosphates per se does not significantly contribute to the overall interaction. This conclusion agrees with the model of sequential multisite binding earlier proposed for visual arrestin-rhodopsin interaction (13). In fact,
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Fig. 4. GRK-independent functional desensitization of β2AR by arrestin2 and 3 mutants. *Xenopus* oocytes were injected with a mixture of cRNAs for the β2AR (0.05 ng), the G protein-gated inwardly rectifying K+ channel subunits Kir3.1 and Kir3.4 (0.02 ng each), and Gs (0.5 ng) that allows the Gs-coupled β2AR to activate the co-expressed channels as described (26). As indicated, some oocytes were also co-injected with 5 ng of cRNA for the different forms of arrestin either alone or together with 0.5 ng of GRK3 cRNA. All recordings were performed 3–4 days postinjection. Receptor-activated currents were measured in 16 mM K+ buffer (26) at −80 mV holding potential. The agonist-elicited responses were adjusted by baseline subtraction as described (26) and normalized to the peak response. Average peak β2AR responses with different arrestin mutants were not significantly different compared with β2AR responses with the parental WT arrestins. A, representative traces depicting β2AR-activated current responses elicited by 1 μM agonist isoproterenol and reversed by 1 μM antagonist propranolol. Antagonist perfusion was used to determine the amount of residual receptor response. The short vertical lines through the traces indicate when agonist treatment was discontinued and the antagonist perfusion started. Calibration scales are the same for each trace (2 min). B, the β2AR desensitization rate in each group of oocytes is expressed as a multiple of the desensitization rate in the oocyte group injected with cRNA for the corresponding WT arrestin only. *, p < 0.05; Student’s t test, compared with the desensitization rate of the corresponding group co-expressing the parental WT arrestin. Each bar represents the mean ± S.E. of from four to eleven separate oocytes.

Fig. 5. GRK-independent functional desensitization of DOR by arrestin2 and 3 mutants. *Xenopus* oocyte injections and methods were as described in the legend to Fig. 4, except that 0.4 ng of DOR cRNA was used instead of β2AR cRNA, and the responses were elicited with the specific peptide DOR agonist DPDPE. Average peak DOR responses with different arrestin mutants were not significantly different compared with DOR responses with parental WT arrestins. A, representative traces depicting DOR-activated current responses elicited by 1 μM DOR agonist DPDPE and reversed by 1 μM antagonist naloxone. Antagonist perfusion was used to determine the amount of residual receptor response and is indicated by the short vertical lines through the traces. Calibration scales are the same for each trace (2 min). B, the DOR desensitization rate in each group of oocytes is expressed as a multiple of the desensitization rate in the oocyte group injected with cRNA for the corresponding WT arrestin only. *, p < 0.05; Student’s t test, compared with the desensitization rate of the corresponding group co-expressing the parental WT arrestin. Each bar represents the mean ± S.E. of from four to eleven separate oocytes.

in oocytes, 30 °C during the binding, and 4 °C during the chromatography in direct binding assay) and the presence of competing molecules (G proteins in oocytes and none in the binding assay). Conceivably, the complex of arrestin3-(1–379) with receptor is stable enough to form in the absence of competition and survive a few minutes at 4 °C on minicolumns (see “Experimental Procedures”) but not stable enough to compete out G protein at 25 °C.

To ascertain whether phosphorylation-independent forms of non-visual arrestins retain receptor specificity of the parental proteins (1, 2, 9, 16, 17), we tested the ability of these forms to promote desensitization of another GPCR, the δ-opioid receptor. The mutants that show constitutive activity toward β2AR also desensitize DOR in a phosphorylation-independent fashion (Fig. 5). All arrestins tested (including WT arrestin2, WT arrestin3, and arrestin3-(3–393)) that desensitize only phosphorylated β2AR effectively shut off DOR signaling in the presence of GRK3. Thus, structurally diverse mutations yielding phosphorylation-independent forms of both non-visual arrestins do not appear to affect their receptor specificity, i.e. a clear preference for β2AR and DOR over rhodopsin (Figs. 2–5).
Receptor phosphorylation serves as a universal trigger for arrestin binding. However, the sequential multisite model of arrestin-receptor interaction (13) predicts that, in the resulting high affinity receptor-arrestin complex, direct arrestin interaction with receptor-attached phosphates provides a relatively small proportion of the binding energy. This prediction is borne out by the observation that phosphorylation-independent mutants of visual arrestin (11, 12, 14, 25) and arrestin2 (17, 18) bind with high affinity to unphosphorylated receptors and to truncated forms of their cognate receptors lacking the C-terminal domain previously shown to contain the phosphorylation sites required for GRK/arrestin-dependent desensitization (26, 31). To test whether this is also true for arrestin3 mutants, we co-expressed them with truncated DOR-(1-339) (18). In sharp contrast to WT DOR (Fig. 5), the rate of desensitization of this receptor cannot be substantially accelerated by arrestin3+GRK3 (Fig. 6). However, arrestin3-(1-392) and arrestin3-(R170E) both accelerate DOR-(1-339) desensitization 2.5–3.5-fold, i.e. to the same extent as WT arrestin3+GRK3 accelerate the desensitization of WT DOR (compare Figs. 5 and 6). Thus, when arrestin3 is activated by appropriate mutations, it does not require either phosphates or the phosphorylation sites themselves for high affinity binding to the receptor.

DISCUSSION

Within a relatively small family of four vertebrate arrestins, overall residue conservation is fairly modest (50–60% identity) (19, 22). However, five charged residues comprising the polar core (homologs of Arg-175, Arg-382, Asp-30, Asp-296, and Asp-303 in bovine visual arrestin) as well as groups of bulky hydrophobic residues in all participants of the three-element interaction are remarkably conserved throughout the family, even in invertebrate arrestins (19). This conservation suggests the important role of these residues in arrestin function, which is supported by the residues’ participation in key stabilizing interactions in the free (inactive) state of visual arrestin (19) and arrestin2 (32). This notion is further strengthened by the dramatic changes in arrestin binding characteristics due to mutations in these two hot spots (10–19, 25). The consequences of these mutations can be easily rationalized in the context of the sequential multisite model of arrestin-receptor interaction (13).

Interpreted in the context of the crystal structure, this model predicts that stabilizing intramolecular interactions are sequentially broken by an active phosphoreceptor, which allows arrestin to undergo the significant conformational rearrangement necessary for its transition into a high affinity receptor binding state (19, 24). In the case of visual arrestin, this prediction was corroborated by extensive structure-based mutagenesis (15, 25).

Here we demonstrate that homologous mutations in both non-visual arrestins yield proteins that bind with high affinity to unphosphorylated receptors (Figs. 2 and 3) and effectively desensitize them in a phosphorylation-independent fashion (Figs. 4–6). These results strongly suggest that the activation mechanism used by all three arrestins is essentially the same, implying that the overall three-dimensional structure of both non-visual arrestins and the conformational rearrangements necessary for receptor binding are very similar to those of visual arrestin. Mutations in both hot spots in all arrestins appear to loosen up their basal conformation, thus making the transition into the binding-competent state easier, as evidenced by the higher binding of the mutants. This effect is most pronounced when the binding of the parental WT protein is relatively low, i.e. in case of binding to an active unphosphorylated receptor (62AR* or Rh*), an inactive phosphoreceptor (P-β2AR or P-Rh), or the wrong active phosphoreceptor (visual arrestin to P-β2AR* and arrestins 2 and 3 to P-Rh*; see Figs. 2 and 3).

The polar core and three-element interaction in visual arrestin are both sequentially destabilized by receptor-attached phosphates (15, 19, 25). First, the phosphates encounter two highly exposed positive charges on the rim of the N-domain bowl (Lys-14 and Lys-15; here and below we use residue numbers for bovine visual arrestin). These adjacent lysines are localized on the β-strand I so that the long side chains point in the opposite directions (19). Phosphate binding likely forces Lys-14 to flip over toward the cavity of the bowl, thereby destabilizing the short β-strand I (25). The three preceding residues on this β-strand are Val-11, Ile-12, and Phe-13, i.e. the three bulky hydrophobic residues that interact with the α-helix I and β-strand XX of the C-tail (19, 25). Thus, destabilization of β-strand I disrupts the three-element interaction and allows Lys-14 and Lys-15 to move with the phosphates into the bowl formed by the N-domain guiding the phosphates toward the main phosphate sensor, Arg-175 of the polar core (25). The presence of the two phosphate binding elements in arrestin, each controlling one of the two key stabilizing intramolecular interactions, is the reason why arrestins in which either one is disrupted by mutagenesis still respond to receptor phosphorylation status, e.g. bind inactive phosphorylated receptor.

For high affinity binding, arrestins require both receptor phosphorylation and activation (10–25). The localization of arrestins’ putative “activation-recognition” site (13) and the mechanism of its function remain to be elucidated. The binding of phosphorylation-independent mutants of the three arrestins to unphosphorylated receptors is triggered solely by receptor activation (Figs. 2 and 3; Refs. 10–19). Thus, this behavior allows us to draw certain inferences regarding the activation-recognition function of arrestin proteins. The most striking feature of these mutants is that their binding to unphosphorylated receptors is more sensitive to the nature of the receptor than the binding of WT or mutant forms of arrestins to phosphorylated/activated receptors (Figs. 2 and 3). Mutants of both non-visual arrestins bind to unphosphorylated 62AR* but not to Rh*, whereas mutants of visual arrestin demonstrate the opposite receptor preference (Figs. 2 and 3; see also Refs. 16 and 18). These data lead us to conclude that activation-recog-
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For preactivated mutants, a weaker push provided by receptor-attached phosphates and the active state of the right receptor to assume the receptor binding conformation. For preactivated mutants, a weaker push provided by phosphates alone, activation alone, or an active state of the wrong phosphoreceptor is sufficient. However, the mutants do not bind appreciably to unphosphorylated inactive receptors or to the wrong activated unphosphorylated receptors (that cannot provide any push at all) (Fig. 3), suggesting that the energy barrier is not reduced to zero even in the most potent constitutively active mutants.

The conservation of both phosphate-sensitive hot spots throughout the arrestin family also suggests that all arrestins require the same receptor phosphorylation levels for high affinity binding. Both direct binding assay in vitro (13, 16, 25) and experimentation with rhodopsin with a limited number of phosphorylation sites in transgenic mice (28) indicate that more than two phosphates per receptor are necessary for productive arrestin interaction.

GPCRs are the most ubiquitous class of sensor molecules in eukaryotic cells. GPCR signaling regulates countless biological processes, and various disturbances in the signaling itself or in its attenuation by the GRK-arrestin pathway result in a wide array of pathological conditions (reviewed in Refs. 29 and 30). We believe that phosphorylation-independent versions of arrestin proteins will serve as useful tools for manipulation of the efficiency of GPCR signaling and possibly for gene therapy of the disorders associated with its ineffective regulation in vivo.

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