Phosphorylation-independent Suppression of Light-activated Visual Pigment by Arrestin in Carp Rods and Cones*

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Background: Photoreceptor arrestin is thought to act on light-activated (R*) and phosphorylated visual pigment to completely suppress the R* activity.

Results: Both rod and cone arrestins bind directly and transiently to nonphosphorylated R* after light and suppressed its activity.

Conclusion: Arrestin suppresses the R* activity without pigment phosphorylation.

Significance: Novel mechanism of arrestin effect is found.

Visual pigment in photoreceptors is activated by light. Activated visual pigment (R*) is believed to be inactivated by phosphorylation of R* with subsequent binding of arrestin. There are two types of photoreceptors, rods and cones, in the vertebrate retina, and they express different subtypes of arrestin, rod and cone type. To understand the difference in the function between rod- and cone-type arrestin, we first identified the subtype of arrestins expressed in rods and cones in carp retina. We found that two rod-type arrestsins, rArr1 and rArr2, are co-expressed in a rod and that a cone-type arrestin, cArr1, is expressed in blue- and UV-sensitive cones; the other cone-type arrestin, cArr2, is expressed in red- and green-sensitive cones. We quantified each arrestin subtype and estimated its concentration in the outer segment of a rod or a cone in the dark; they were ~0.25 mM (rArr1 plus rArr2) in a rod and 0.6 – 0.8 mM (cArr1 or cArr2) in a cone. The effect of each arrestin was examined. In contrast to previous studies, both rod and cone arrestins suppressed the activation of transducin in the absence of visual pigment phosphorylation, and all of the arrestins examined (rArr1, rArr2, and cArr2) bound transiently to most probably nonphosphorylated R*. One rod arrestin, rArr2, bound firmly to phosphorylated pigment, and the other two, rArr1 and cArr2, once bound to phosphorylated R* but dissociated from it during incubation. Our results suggested a novel mechanism of arrestin effect on the suppression of the R* activity in both rods and cones.

In the vertebrate retina, there are two types of photoreceptors, rods and cones. In both cells, light activates visual pigment, and light-activated visual pigment (R*) activates a GTP-bind-

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‡ The abbreviations used are: R*, activated visual pigment; Tr, transducin; Tr* activated transducin; PDE, cGMP phosphodiesterase; GRK, G-protein-coupled receptor kinase; OS, outer segment; IS, inner segment; GTP-γs, guanosine-5’-[(γ-thio)triphosphate; AMPPNP, adenylyl-imidodiphosphate; Arr, arrestin; VP, visual pigment.  

Significant:

Novel mechanism of arrestin effect is found.
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EXPERIMENTAL PROCEDURES

Preparation of Rod and Cone Membranes—C. carpio rods and cones were purified in the dark, and their membranes were prepared as described previously in complete darkness with the aid of an IR converter (NVR 2015, NEC) (13). Measurements of activation of Tr and cone PDE activity were also carried out in complete darkness. Animals were cared for according to the institutional guidelines (approval number, FBS-14-005). The content of visual pigment in purified rods and cones was determined spectrophotometrically as described previously (15). In both of our rod or cone membranes used in this study, GRK and Tr levels were similar to those in the membranes we usually use for biochemical measurements of reactions in the phototransduction cascade, and no detectable protein elution from these membranes was observed in the presence of arrestins and ATP in the dark.

Expression and Purification of Recombinant Carp Arrestins—
All carp arrestins, including rod arrestins, rArr1 (AB794063) and rArr2 (AB794064), and cone arrestins, cArr1 (AB794065) and cArr2 (AB794066), were obtained from carp retinal cDNA library (16). Each of the coding regions of carp rod arrestins, rArr1 or rArr2, was cloned into the Ncol/Xhol sites in pET-16b (Novagen). Each of the coding regions of carp cone arrestins, cArr1 or cArr2, was cloned into the Ncol/Xhol or Ncol/BamHI sites in pTrcHisC (Invitrogen). These recombinant plasmids were introduced into Escherichia coli BL21 (DE3).

Expression and purification of arrestins were carried out based on the method described previously (17). The cells expressing recombinant arrestins were collected and sonicated in Tris/EGTA buffer (50 mM Tris-HCl, 5 mM EGTA, pH 8.0). Rod arrestins, rArr1 and rArr2, were obtained in the soluble fraction, and cone arrestins, cArr1 and cArr2, were mainly found in the insoluble inclusion bodies. Rod arrestins in soluble fractions were first roughly purified through ammonium sulfate precipitation and further purified by heparin-Sepharose 6 Fast Flow column (GE Healthcare). The fractions containing rod arrestins were mainly eluted at 350 mM NaCl in column buffer (10 mM Tris-HCl, 0–500 mM NaCl, 2 mM EGTA, 2 mM EDTA, pH 7.5). Cone arrestins in insoluble fractions were solubilized with Tris/EGTA buffer containing 8 mM urea, dialyzed against column buffer devoid of NaCl, and then purified by the heparin-Sepharose column (GE Healthcare). Fractions containing cone arrestins were eluted at 350 mM NaCl in column buffer. Further purification by Q-Sepharose fast flow column (GE Healthcare) was carried out when necessary. Fractions containing each arrestin were concentrated and buffer-exchanged to potassium glutonate buffer (K-gluc buffer: 115 mM potassium glutonate, 10 mM HEPES, 2.5 mM KCl, 2 mM MgCl2, 0.2 mM EGTA, 0.1 mM CaCl2, 1 mM dithiothreitol, pH 7.5) using Vivasin 20 (GE Healthcare). Recombinant arrestins were used in our biochemical study. In our control studies using endogenous arrestins obtained from purified rods and cones, rod (rArr1 and rArr2) and cone (mainly cArr2) arrestins showed transient binding to rod and cone membranes, respectively, in the absence of visual pigment phosphorylation as our recombinant rod and cone arrestins do (see under “Results”), which indicated that our recombinant arrestins are physiologically functional. The amount of purified recombinant arrestin was quantified with Coomassie Brilliant Blue staining after SDS-PAGE, using bovine serum albumin as a molar standard.

Preparation of Polyclonal Antisera—Partial sequences of rArr1, rArr2, cArr1, and cArr2 genes correspond to amino acid residues 353–385, 353–390, 66–102, and 68–110, respectively, were inserted into the EcoRI/Xhol sites in pGEX 5 X-1 vectors (Invitrogen). N-terminal GST fusion peptides of arrestins coded by these vectors were expressed in E. coli BL21 cells and purified using a glutathione resin column (GE Healthcare) according to the manufacturer’s protocol. Mice were immunized with these partial peptides to raise anti-rArr1, anti-rArr2, anti-cArr1, and anti-cArr2 antisera. The antisera obtained from anti-rArr1 and anti-rArr2 were slightly cross-reactive with each other. Cross-reactivities between anti-rArr1 and anti-rArr2 antisera were removed by absorption with recombinant rArr2 and rArr1, respectively.

Immunohistochemical Identification of Co-localization of the Visual Pigment Subtype and Arrestin Subtype—Carp were light-adapted for 3 h before the retinas were removed. The retinas dissected in the light were immediately immersed in 100 mM phosphate buffer (PB: 19 mM NaH2PO4, 81 mM Na2HPO4, pH 7.4) containing 5% (w/v) sucrose and 4% (w/v) paraformaldehyde and fixed at 4 °C for 2 days (16). Then they were embedded in 33% (w/v) OCT compound (Sakura Finetec) diluted with PB containing 20% (w/v) sucrose. The 6-μm-thick cryosections were mounted on maminopropylsilane-coated slides and preincubated in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM NaH2PO4, pH 7.4) containing 10% (v/v) normal goat serum at 25 °C for 1 h. After washing with PBS three times, retinas were incubated with mouse anti-rArr1, anti-rArr2, anti-cArr1, or anti-cArr2 antisera (all at 1:1000 dilution) overnight at 4 °C. Each type of cone was labeled with specific antiserum raised in rabbit as follows: anti-red/green-sensitive cone opsins antiserum raised against a peptide of CSTKSKEVSSVAPA in red-sensitive cone opsin (AB055656.1); green-sensitive cone opsin1 and -2 (AB110602.2 and AB110603.1, respectively) (1:2000 dilution); anti-blue-sensitive cone opsin antiserum raised against a peptide of CGYAEDTNKDYR in UV-sensitive opsin (AB113668.1) (1:1000 dilution); anti-UV-sensitive cone opsin antiserum raised against a peptide of CGYAEKTNKDYR in UV-sensitive opsin (AB113669.1) (1:500 dilution). (Our anti-red/green-sensitive cone opsin antiserum reacts to both red- and green-sensitive cone opsins.) Immunoreactivities against arrestins were detected by anti-mouse IgG labeled with fluorescein isothiocyanate, and those against rod or cone pigments were detected by anti-rabbit IgG labeled with Texas Red (Vector Laboratories).

Quantification of Endogenous Arrestins—To determine the expression level of each arrestin in carp rods and cones, we quantified spectrophotometrically the total content of rod pigment and red-sensitive cone pigment in a retinal homogenate. In carp retina, rods are more abundant than cones by ~50 times (13), so the visual pigment is mostly rod pigment in a retinal homogenate. In an aliquot of a homogenate, first we spectrophotometrically measured the content of red-sensitive cone pigment with bleaching by >660 nm light (15). From our previous estimation of the ratio of the amount of each cone pig-
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Because visual pigment is present exclusively in the OS, this immunofluorescently determined fraction of arrestin in the OS gives us the amount of each arrestin in the OS of the OS-IS rods from the above. This quantitative analysis, and also the amount of each visual pigment, was quantified spectrophotometrically (15). Then the apparent immunoblot analysis, and also the amount of each visual pigment was quantified spectrophotometrically (15). Then the fraction of arrestin present in the OS in a single isolated dark-adapted OS-IS cell prepared separately was immunofluorescently quantified (see below). Because the total amount of each arrestin in the preparation of OS-IS rods or cones is known from the above, this immunofluorescently determined fraction gives us the amount of each arrestin in the OS of the OS-IS rods or OS-IS cones. Because visual pigment is present exclusively in the OS and its concentration is assumed to be 3 mM (18), we can estimate the concentration of each arrestin in the OS in the dark from the ratio of the amount of arrestin in the OS to that of visual pigment.

Immunofluorescent Staining of OS-IS Cells—Immunofluorescent staining of OS-IS rods and cones was performed as described (19). Carp rods and cones were obtained in the dark with a simplified purification method by using a stepwise Percoll density gradient consisting of 30 and 90% Percoll in the presence of 4% (w/v) paraformaldehyde. Both cells were obtained at the interface between the layers of 30 and 90% Percoll. The cells were washed twice with carp Ringer’s solution (119.9 mM NaCl, 2.6 mM KCl, 0.5 mM CaCl2, 0.5 mM MgCl2, 0.5 mM MgSO4, 1 mM NaHCO3, 16 mM glucose, 0.5 mM NaH2PO4, 4 mM HEPES, pH 7.5) supplemented with 4% (w/v) paraformaldehyde and then incubated in the same buffer at 4 °C for 12 h in the dark. Then they were mounted on an aminopropylsilane-coated slide glass. Mounted OS-IS rods were permeabilized with 0.01% (w/v) Triton X-100 in PBS and mounted OS-IS cones with 0.2% (w/v) Triton X-100 in PBS. Normal goat serum was added in the permeabilization process to reduce nonspecific absorption of antiserum. After washing with PBS three times, OS-IS rods were incubated overnight at 4 °C with anti-rArr1 or anti-rArr2 antiserum (both at 1:1000 dilution). OS-IS cones were incubated overnight at 4 °C with anti-blue-sensitive cone opsin antiserum (1:1000 dilution) plus anti-cArr1 (1:200 dilution), anti-UV-sensitive cone opsin antiserum (1:1000 dilution) plus anti-cArr1 (1:200 dilution), or anti-red/green cone opsin antiserum (1:1000 dilution) plus anti-cArr2 (1:200 dilution). OS-IS rods and cones were then washed with PBS three times and incubated at 25 °C for 30 min with anti-mouse IgG conjugated with fluorescein isothiocyanate to detect arrestin, and with anti-rabbit IgG conjugated with Texas Red to detect cone pigment subtype. Immunofluorescent signals of arrestins in the OS relative to those in an entire OS-IS cell were quantified to estimate the relative content of an arrestin in the OS in an OS-IS rod or OS-IS cone. In this quantification, signal intensity in each part (entire cell, the OS, or the IS) in an immunofluorescent image was quantified using ImageGauge software (version 4.0, Fuji Film).

Quantification of Tr*—The amount of Tr* formed was quantified by measuring the amount of GTPγS bound to Tr with a filter binding assay as described previously (20). Rod or cone membranes suspended in K-gluc buffer were preincubated for 30 s in 15 μl of a reaction mixture containing 1 μM visual pigment, 0–60 μM recombinant arrestin or ovalbumin, 100 μM [35S]GTPγS, 100 μM GDP, 0.8 mM EGTA, 10 mM UDP, and when necessary 1 mM ATP. When the effect of arrestin was examined, ovalbumin was used as a control protein. We examined several proteins as a control, and all of them affected the GTPγS binding to Tr to some extent. Among them, ovalbumin was the most ineffective protein, although it inhibited the GTPγS binding to ~90% of the binding observed in the absence of added proteins. Our rod and cone membrane preparations contain membranes derived from the IS of rods and cones, respectively. Because the size of the IS is larger than that of the OS in our purified cones, metabolic enzyme activities such as ATPase and GTP synthetase are contained. In this case, GDP added to inhibit the nonspecific binding of GTPγS (21) can be regenerated to GTP with hydrolysis of ATP when it is present. The product, GTP, is utilized to activate Tr. In this case, in our [35S]GTPγS binding assay we cannot measure the amount of Tr* correctly. For this reason, UDP was added to inhibit GTP synthesis (22) induced by added ATP and GDP. UDP also partially inhibited the rate of activation of Tr in rod and cone membranes probably by competitive inhibition with GTPγS, but the maximum binding of GTPγS was unaffected both in rod and cone membranes. After the reaction mixture was illuminated with a light flash bleaching 0.03 and 0.3% of visual pigment in rod and cone membranes, respectively. Because the size of the IS is larger than that of the OS in our purified cones, metabolic enzyme activities such as ATPase and GTP synthetase are contained. In this case, GDP added to inhibit the nonspecific binding of GTPγS (21) can be regenerated to GTP with hydrolysis of ATP when it is present. The product, GTP, is utilized to activate Tr. In this case, in our [35S]GTPγS binding assay we cannot measure the amount of Tr* correctly. For this reason, UDP was added to inhibit GTP synthesis (22) induced by added ATP and GDP. UDP also partially inhibited the rate of activation of Tr in rod and cone membranes probably by competitive inhibition with GTPγS, but the maximum binding of GTPγS was unaffected both in rod and cone membranes. After the reaction mixture was illuminated with a light flash bleaching 0.03 and 0.3% of visual pigment in rod and cone membranes, respectively. GTPγS binding time course was measured.

To examine the dose dependence of the suppression by an arrestin subtype on Tr activation, light flashes bleaching 0.02 and 1.2% of visual pigment in rod and cone membranes, respectively, were used. The flash intensities in this study were determined to activate similar amounts of Tr* (~65% of total Tr) in rod and cone membranes; due to a lower production rate of Tr* and shorter lifetimes of R* in cones than in rods, cones require much more intense light than rods to produce a similar amount of Tr* (21). The injection of [35S]GTPγS was terminated at various time intervals after a light flash by adding 10–20 times volume of a quench solution, K-gluc buffer containing 50 mM NH4OH and 25 mM cold GTP, to the reaction mixture. The
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timing of addition was strictly controlled using a rapid-quench apparatus (15). To quantify the amount of Tr* produced by a light flash, GTPγS binding in the dark was subtracted. All measurements were carried out at 20 °C throughout.

Quantification of Arrestin Bound to Rod or Cone Membranes—Binding of arrestin to rod or cone membranes was measured based on a binding assay method described previously (23). For the study in rod membranes, the membranes were incubated for 10 min in the dark or under bright illumination at room temperature in 40 μl of K-gluc buffer containing 0.625 μM visual pigment, 0.625 mM GTP, and 0.95 mM EGTA in the presence or absence of 1.25 mM ATP, and then 10 μl of K-gluc buffer containing 1 μM arrestin was added. After 2 min of incubation in the dark or light, the membranes were precipitated by centrifugation at 124,000 × g for 10 min. The amount of arrestin in the precipitate was quantified by quantitative immunoblot analysis. To estimate nonspecific arrestin binding to rod membranes, liposomes were prepared with lipids extracted from rod membranes with the Bligh and Dyer extraction method (24), and arrestin binding to the liposomes containing equivalent amounts of phospholipids in the rod membrane samples (5 nmol of phospholipids) was measured and subtracted. Binding to control liposomes was 0.01–0.02 molecules of arrestin per pigment present when the binding was estimated in terms of per pigment present in rod membranes. Quantification of phospholipids was made as reported previously (25).

The binding of arrestin to cone membranes in the dark and under bright illumination was measured similarly as in rod membranes. For cones, their availability is limited, so that preparation of liposomes with lipids obtained from purified cones was not possible. For this reason, we could not eliminate the background nonspecific binding of cone arrestin to the cone membranes in this estimation.

In this study, we also measured the time course of the binding of arrestin to rod or cone membranes after a light flash. When the time course of rArr1 or rArr2 binding to rod membranes was measured, a light flash bleaching 6% of the pigment was given to rod membranes in the presence of either of rod arrestins and in the presence and absence of ATP. The membranes were incubated for the desired time periods at room temperature in the dark, and then they were immediately (≤5 s) transferred to a centrifuge rotor chilled at 4 °C and centrifuged briefly (17,860 × g, min) at 4 °C. After removing the supernatant quickly, 40 μl of SDS sample buffer (50 mM Tris-HCl, 2% (w/v) SDS, 10% (w/v) glycerol, 0.0025% bromphenol blue, 1.25% (w/v) 2-mercaptoethanol, pH 6.8) was added to the precipitate to quantify both the pigment and the bound arrestin with immunoblot. In our binding time course measurement shown in Fig. 5B, the time point indicates the length of the time of incubation at room temperature in the dark.

Time course of cArr2 binding to cone membranes was measured first at room temperature similarly as in rod membranes, but we did not find significant light-dependent binding of cArr2 to cone membranes either in the presence or absence of ATP. It was probably because transient binding of cArr2 to cone membranes was too fast in our binding measurement. For this reason, we measured the cArr2 binding to cone membranes at 4 °C. A light flash bleaching 30% of the pigment was given to cone membranes.

Binding of both rod and cone arrestins to membranes is probably because of the binding to R*. We confirmed that the arrestin binding to the membranes increased light-dependently and that the binding is proportional to the amount of bleached pigment, R*.

Measurement of Cone PDE Activity in the Dark—PDE activity was measured with the pH assay method as described previously (21). Cone membranes suspended in K-gluc buffer containing 0.8 mM EGTA were first mixed with cGMP, and then arrestin or ovalbumin in K-gluc buffer was added. The measurement of PDE activity in the dark was initiated by adding GTP. When necessary, ATP was added 4 min before the addition of GTP. In the absence of ATP, we added the same volume of K-gluc buffer to the mixture as a control. The final volume in the mixture was 100 μl, and the ingredients in the mixture were 1 μM visual pigment, 30 μM cArr2 or ovalbumin, 0.5 mM GTP, 5 mM cGMP, and 0.8 mM EGTA, and when necessary 1 mM ATP. To measure the maximum PDE activity, samples were exposed to a bright light flash at the end of each measurement. All manipulations were carried out at room temperature.

RESULTS

Identification and Localization of Arrestins in Carp Rods and Cones—To examine the difference of the effect of arrestins on the phototransduction cascade between rods and cones, we first identified arrestins expressed in rods and cones in carp. In agreement with previous studies in zebrafish (11), we found four orthologs of arrestin in our carp retinal cDNA library (26). Two of them were rod-type arrestins, rArr1 (AB794063) and rArr2 (AB794064), that were named after medaka rod arrestins (27), and two of them were cone-type arrestins, cArr1 (AB794065) and cArr2 (AB794066). Based on a study in zebrafish (11), amino acid sequence identities are 90% between carp rArr1 and zebrafish arr5b, 87% between rArr2 and arr5a (NM_200559.1), 88% between cArr1 and arr3b (NM_200792.1), and 92% between cArr2 and arr3a (NM_001002405.1). To examine localization of each of the carp arrestins, we raised specific antiserum against each of carp arrestins (Fig. 1A). As reported previously (11), our immunoblot studies on purified rods and cones (Fig. 1B) and immunohistochemical studies (Fig. 1, C and D) confirmed the following: 1) rod-type arrestins (rArr1 and rArr2) are expressed in rods (Fig. 1B); 2) cArr1 is expressed in blue- and UV-sensitive cones (arrowheads in the immunofluorescent images in a pair of left and middle panels in Fig. 1C) but not in red/green-sensitive cones (thick arrows in a pair of right panels in Fig. 1C); and 3) cArr2 is expressed in red/green-sensitive cones (arrowheads in a pair of right panels in Fig. 1D) but not in blue- and UV-sensitive cones (thick arrows in a pair of left and middle panels in Fig. 1D). When rods were reacted with anti-rArr1 or anti-rArr2 antisera, all of the rods were stained with either of the antisera. This result indicated that rArr1 and rArr2 are co-expressed in a rod. Cone cell type-dependent expression pattern of cArr1 and cArr2 is similar to that reported for arr3b and arr3a, respectively, in zebrafish (11).
Expression Levels of Arrestins in Rods and Cones—Arrestins are found in the OS, in the IS, in the nuclear region, and in the synaptic terminal in the dark, and they move toward the OS in the light in both rods (27, 28) and cones (29, 30). To determine the expression levels of each arrestin in a rod or a cone, we first quantified the total amount of each arrestin in a carp retinal homogenate. As shown in the left panel in Fig. 2A, with immunoblot, the amount of each arrestin in a retinal homogenate (retina) was compared with that of the corresponding recombinant protein of known amounts, and the amount of arrestin in the homogenate was determined from a calibration line obtained in the immunoblot (right panel; only the result for quantification of cArr1 is shown). We also quantified or estimated the amount of each of the pigments in the same carp retinal homogenate and determined the ratio of the amount of each arrestin relative to that of the visual pigment co-expressed. The ratio of the amount of arrestin to that of visual pigment (Arr/VP) is an indication of the amount of arrestin expressed in each arrestin relative to that of the visual pigment co-expressed.

Because we wanted to know their effects on R* when a light flash is given in the dark, we tried to estimate the concentration of arrestins in the OS in rods and cones in carp under dark-adapted conditions. For this purpose, we used purified rods and cones. These cells consist of the OS and the IS but lack the nucleus and the terminal regions. In this section and below, we call these cells OS-IS rods or OS-IS cones to help with understanding the procedure (see “Experimental Procedures”). In this preparation, we quantified the amount of each of the arrestins in OS-IS rods or cones by immunoblot with specific antiserum, using recombinant arrestin as a molar standard (rod or cone in the left panel in Fig. 2A). From the calibration line obtained, we quantified the amount of each arrestin in OS-IS rods and cones (cone in the right panel in Fig. 2A for quantification of cone cArr1 in OS-IS cones). We also measured the quantity of each of the pigment in OS-IS rods and cones. From the quantities of arrestin and the pigment co-expressed, we obtained an Arr/VP ratio in each cell type of OS-IS rods or cones (Table 2A).

Relative abundance of arrestin in the OS in an OS-IS cell could be determined from relative intensities of immunofluorescent signals in the OS of an isolated single OS-IS cell (Fig. 2, B for rods and C for cones), and the obtained result for each of
arrestins is shown in Table 2B. This portion of arrestin is present together with the visual pigment of which the concentration is 3 mM in the OS in both rods and cones (18). Under the assumption that the cytoplasmic volume of an OS is 1/2 of the OS envelope volume (31), we estimated the arrestin concentrations in rods and in each type of cone in carp retina (Table 2C). It was found that the concentration of arrestin in the OS in the dark-adapted state is ~0.25 mM in rods (rArr1 plus rArr2) and 0.6 – 0.8 mM in blue/UV-sensitive cones (cArr1) and in red/green-sensitive cones (cArr2). The concentration of arrestin in the dark is 3–4 times higher in the cone OS than in the rod OS.

**Effect of Arrestins on Transducin Activation in Rod and Cone Membranes**—To examine whether R* lifetime is affected by arrestin differently in rods and cones, we compared Tr activation time course between rod membranes containing rod arrestin and cone membranes containing cone arrestin at an arrestin concentration of 30 nM. This concentration of arrestin was well below the estimated arrestin concentration in the OS in the dark both in rods and cones (Table 2C), but it was the second highest concentration we could prepare in this study. In the measurement in rod membranes, we used rArr1 or rArr2. In the measurement of cone membranes, we used only cArr2.
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Expressed in red- and green-sensitive cones, because 80% of cones in our purified cone preparation are red- and green-sensitive cones (13). The effect of arrestin was examined in the absence and presence of ATP to evaluate the difference of the arrestin effect on nonphosphorylated and phosphorylated R*.

In Fig. 3A, Tr activation was measured as the binding of GTPyS to Tr per R* after a light flash. When only ATP was added in Fig. 3A (filled black circles), initial Tr activation did not seem to be affected significantly (see “Discussion”; early time courses of the effect of rArr1 together with that of cArr2 are shown in Fig. 3F). At several seconds after a light flash in Fig. 3A, Tr* formation was suppressed when compared with the Tr* formation without ATP (open black circles). This suppression would be due to the quenching of R* by pigment phosphorylation. There might be a possibility that ATP acted on Tr to suppress Tr* formation. This possibility is excluded because Tr was not phosphorylated in the presence of ATP and because a non-hydrolyzable ATP analogue, AMPPNP, did not show suppression of Tr* formation.

When only rArr1 was added (open red circles) in Fig. 3A, initial Tr* formation did not seem to be affected either, but Tr* formation was suppressed several seconds after the flash similarly as in the case where ATP was added (filled black circles). This result is surprising, because it has been believed that arrestin does not affect R* activity when R* is not phosphorylated (4, 5). On the contrary to this belief, our result clearly showed that nonphosphorylated R* is quenched by arrestin at least in carp. The extent of the suppression by rArr1 was even larger than that by ATP under the condition employed in Fig. 3A. There might be a possibility that the pigments we used were phosphorylated already in the dark-adapted animal, so that the added arrestin bound to phosphorylated R*. This would not be the case. In a previous study (32), it has been shown that only ~1% of the visual pigment is phosphorylated in both rods and cones in dark-adapted mice and zebrafish, respectively. This situation would be applicable to our dark-adapted carp. Then it is possible that ~1% of visual pigment had been phosphorylated already in our membranes, but it is difficult to explain a much larger effect of rArr1 on Tr* formation (~80%); compare the steady levels among those shown with open black circles, open red circles, and filled red circles in Fig. 3A).

When both rArr1 and ATP were added, initial Tr* formation did not seem to be affected significantly, but the suppression of Tr* formation was the greatest in these four types of measurements. The result indicates that the suppressive effect by ATP plus rArr1 is higher than that by ATP or rArr1 alone. Because the amount of transducin was similar in each of the membranes used in a group of the study shown in Fig. 3A (see “Experimental Procedures”), suppression of Tr* formation in the presence of ATP and/or rArr1 was not due to a loss of transducin.

Although the extent of the suppression by arrestin and that by ATP were different depending on what arrestin was added and which membranes were used, the suppression on nonphosphorylated R* by arrestin alone and the higher effect of arrestin plus ATP were observed in all of the measurements in rod membranes (Fig. 3, A and B) and in cone membranes (Fig. 3D). The amounts of Tr* formed at 60 s after the light flash in rod membranes and that at 5 s in cone membranes are shown in Fig. 3, C and E, respectively.

Fig. 4 shows dose dependence of the effect of arrestin on Tr* formation at 60 s after a light flash in rod membranes (Fig. 4, A and B) and at 5 s in cone membranes (Fig. 4C). In these measurements, flash intensities were attenuated to produce a similar amount of Tr* in rod and cone membranes (see “Experimental Procedures”). In agreement with the results shown in Fig. 3, rArr1, rArr2, and cArr2 all suppressed Tr* formation in the absence of ATP (open circles in Fig. 4), and the suppression was higher when ATP was added (filled circles). However, the dose dependence was different among the three subtypes of arrestins.
binding of arrestins without visual pigment phosphorylation. To understand their function, we examined (3) that these arrestins suppressed Tr* formation without addition of ATP, which indicated that these arrestins suppressed Tr* formation without visual pigment phosphorylation. To understand their functional mechanisms, we measured the binding of arrestins to rod or cone membranes in the dark and in the light and in the presence and absence of ATP. Fig. 5A shows the binding of rArr1 and rArr2 to rod membranes and that of cArr2 to cone membranes in the dark and in the light and in the presence and absence of ATP. The binding in the light is most due probably to R*, because the binding was dependent on the flash intensity, and therefore the amount of light-dependent GTP

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FIGURE 4. Dose dependence of the suppression of Tr activation by arrestin in the absence and presence of pigment phosphorylation. A and B, dose-dependent effect of arrestin (rArr1 in A and cArr2 in B) was examined by measuring the light-dependent GTP-S binding to rod membranes in the presence of only rod arrestin (open circles) and in the presence of both arrestin and ATP (filled circles) (n = 5). The binding is expressed as the number of GTP-S molecules bound to the membranes per R*. The measurement was made at 60 s after a light flash bleaching 0.02% of the pigment. As controls of addition of arrestin, ovalbumin of the concentration indicated in the abscissa was added. C, dose-dependent effect of cArr2 was examined by measuring the light-dependent GTP-S binding to cone membranes per R* in the presence of only cArr2 (open circles) and in the presence of both cArr2 and ATP (filled circles) (n = 5). The measurement was made at 5 s after a light flash bleaching 1.2% of the pigment. Measurements were made similarly as in A and B. Each data point in A–C indicates mean ± S.E. in n independent measurements.

Rod arrestin, rArr1, suppressed the Tr* formation by increasing its concentration in the range examined (up to 60 μM, Fig. 4A). Because the concentration of rArr1 in the rod OS was estimated to be 150 μM in the dark (Table 2C), its effect in intact cells is probably larger than the maximum suppression observed in Fig. 4A. One noticeable point is that rArr1 alone inhibited the Tr* formation to a level very similar to that attained in the presence of both 60 μM rArr1 and ATP (1 mM). This result suggests that rArr1 alone can suppress Tr* formation to its maximum without phosphorylation. For the other rod arrestin, rArr2, the extent of inhibition of Tr* formation was significantly smaller than that attained by rArr1 (Fig. 4B). Cone arrestin, cArr2, showed a significant suppressive effect on Tr* formation in cone membranes; cArr2 showed almost a saturating suppression on Tr* formation at the lowest concentration we examined (3 μM, Fig. 4C).

Binding of Arrestins to Visual Pigments—It has been generally thought that arrestin binds to phosphorylated visual pigment to completely suppress the R* activity. However, as shown in Figs. 3 and 4, rod arrestins (rArr1 and rArr2) and a cone arrestin (cArr2) all suppressed Tr* formation without addition of ATP, which indicated that these arrestins suppressed Tr* formation without visual pigment phosphorylation. To understand their functional mechanisms, we measured the binding of arrestins to rod or cone membranes in the dark and in the light and in the presence and absence of ATP.

FIGURE 5. Binding of arrestins to rod and cone membranes. A, binding of each arrestin to rod membranes (n = 7 for rArr1 and n = 6 for rArr2) or cone membranes (n = 2 for cArr2) was measured in the presence or absence of ATP in the dark or in the light. The amount of bound arrestin is expressed as the number of arrestin molecules bound per visual pigment present in the membranes used. The result is shown as mean ± S.E. in n independent measurements when n ≥ 3, and mean ± deviation from the mean when n = 2. B, time courses of the binding of rArr1 (top), rArr2 (middle), and cArr2 (bottom) to rod (for rArr1 and rArr2) and cone (for cArr2) membranes after a light flash bleaching 6% of the pigment in rod membranes and 30% in cone membranes with (filled circles) or without (open circles) ATP (n = 3 for rArr1, 8 for rArr2, and 3 for cArr2 in studies both with and without ATP). The binding was measured at room temperature for rod arrestins and at 4 °C for cone cArr2 (see “Experimental Procedures”). Vertical axis shows the amount of arrestin bound per R*. The amount of bound arrestin in the dark was subtracted. Each data point indicates mean ± S.E. in n independent measurements.
R* (see “Experimental Procedures”). In all of these measurements, arrestin binding was the highest in the presence of both ATP and light, although the binding levels were different among arrestin subtypes. This result is in agreement with previous studies (28, 33). However, in all of these measurements in Fig. 5A, arrestin binding in the presence of light without addition of ATP (light, +; ATP, −) was indistinguishable from that measured in membranes kept in the dark without ATP (light, −; ATP, −). These binding data indicate that there are no light-dependent bindings of arrestin in the absence of ATP and are not consistent with the result showing significant suppression of Tr* by arrestin after a light flash in the absence of ATP (open red circles in Fig. 3 and open circles in Fig. 4).

This apparent discrepancy could arise from the difference in the time of the measurement after a light flash; Tr* formation was measured during 60 s in rod membranes or 5 s in cone membranes kept in the dark after a light flash in Figs. 3 and 4, whereas arrestin binding shown in Fig. 5A was measured at the time point when the membranes had been exposed to light for more than 10 min (see “Experimental Procedures”). We therefore examined binding time courses of rArr1, rArr2, and cArr2 in the dark after a light flash by decreasing the time of incubation down to 1 min. As shown in the top and middle panels in Fig. 5B, rod rArr1 and rArr2 both bound to nonphosphorylated R* transiently (1 min or so) and dissociated from it 5 min after the light flash (open circles). In contrast, in the presence of ATP (filled circles), the binding of each arrestin to R* was slightly different between these arrestins. One molecule of rArr1 bound to a phosphorylated R* at 5 min after a light flash but dissociated from it 10 min after the flash (filled circles in the top panel in Fig. 5B). The other rod arrestin, rArr2, bound firmly to R* in the presence of ATP and therefore to phosphorylated R* throughout the period of the measurement (filled circles in the middle panel in Fig. 5B). Although the binding of rod arrestins was measured at 20 °C, the binding of cone arrestin, cArr2, was measured at 4 °C (see “Experimental Procedures”). The binding of cone cArr2 at 4 °C was transient in both the absence and presence of R* phosphorylation (open circles and filled circles, respectively, in the bottom panel in Fig. 5B). The results of binding time course shown in Fig. 5B are consistent with the binding results in Fig. 5A obtained after the membranes were exposed to light for more than 10 min. In other words, the binding of arrestin is dependent on the time after bleaching (see “Discussion”). The amount of cone cArr2 bound to both nonphosphorylated and phosphorylated R* (Fig. 5B, bottom panel) was much smaller than those of rod arrestins (top and middle panels). It is probably because the time resolution of our binding assay was not good enough to detect fast binding time course in cone membranes.

Effect of Arrestin on Activation of Transducin and PDE in the Dark—In this study, we measured Tr* formation in the dark, and this activity was subtracted from the activity after light to estimate the net effect of light on Tr* formation. In these measurements, we realized that Tr* formation in the dark is significantly higher in cone membranes than in rod membranes (Fig. 6A). Without the addition of ATP nor cArr2, 0.024 Tr* per visual pigment present was formed in 30 s in cone membranes in the dark (hatched bar below cone membranes in Fig. 6A), which corresponds to the activity of ~0.0008 Tr* formed per visual pigment present per s. The initial activity of Tr* formation in the light was ~30 Tr* formed per R* per s in the absence of both cArr2 and ATP (Fig. 3D, open black circles). Based on this light-induced activity, with the light flash bleaching 0.003% of the pigment, 0.0009 Tr*/R*/s can be formed. In other words, cone membranes have the activity to form Tr* in the dark equivalent to the activity induced by a light flash bleaching 0.003% of the pigment. Dark activities in rod membranes (bars below rod membranes in Fig. 6A) were ~10 times lower than those in cone membranes (bars below cone membranes). Therefore, the contribution of dark activity in rod membranes is small, but dark activity in cone membranes could be a problem to maintain the light sensitivity in cones. This dark activity in cones is probably due to thermal 11-cis-retinal isomerization of cone pigment (34). If this is the case, the dark activity could be suppressed by ATP and also by cArr2. In fact, the dark activity was suppressed by ATP, and thus R* phosphorylation (gray bar under cone membranes), and also by cArr2 (white bar under cone membranes in Fig. 6A), and the effect of ATP plus cArr2 was higher than that of ATP or cArr2 alone (Fig. 6A, black bar under cone membranes).
The effect of ATP was larger in cones than in rods in the dark (compare the two bars at left under rods membranes with those under cone membranes in Fig. 6A). It is probable that the R* level was higher in cone membranes than in rod membranes in the dark, so the R* phosphorylation effects were larger in cone membranes. In agreement with this notion, although the activities were low, R* phosphorylation rate in the dark was ~50 times higher in cone membranes than in rod membranes in the absence of cArr2: 8.7 ± 2.8 × 10⁻⁴ (mean ± S.E., n = 4) phosphates incorporated (Pi) per visual pigment (R) present per s (Pi/R/s) in cone membranes and 1.7 ± 0.8 × 10⁻⁵ Pi/R/s (n = 3) in rod membranes.

The suppression effect on the Tr* formation by cArr2 and ATP in the dark was examined also at the PDE level. A sample trace recorded in the absence of both ATP and cArr2 (top trace in Fig. 6B) and that recorded in the presence of both ATP and cArr2 (bottom trace in Fig. 6B) are shown. The summarized result (Fig. 6C) shows that PDE dark activity in cone membranes was reduced by both ATP and cArr2 (gray bar and white bar, respectively, in Fig. 6C), and the effect of ATP plus cArr2 was higher than that of ATP or cArr2 alone (black bar in Fig. 6C). These results are consistent with those of the effect of ATP and cArr2 on Tr* formation shown in Fig. 6A.

**DISCUSSION**

In this study, we found that the expression level of arrestin is higher in carp cones than in rods (Tables 1 and 2) and that carp arrestins suppress Tr activation without visual pigment phosphorylation both in rods and cones (Figs. 3 and 4) in contrast to the previous suggestion that arrestin binds to the pigment after phosphorylation. Our binding time course measurements showed that carp rod and cone arrestins transiently bind to nonphosphorylated R* (Fig. 5). Dark cone activity of Tr* formation is potentially high, but it could be suppressed by ATP and cone arrestin (Fig. 6).

**Higher Expression Level of Arrestin in Cones Than in Rods—**In this study in carp retina, we showed that rods express two rod-type arrestins, rArr1 and rArr2, and cones express cone-type arrestins, cArr1 in blue- and UV-sensitive cones and cArr2 in red- and green-sensitive cones (Fig. 1). This localization of arrestin subtype is similar to those in zebrafish (carp cArr1 as zebrafish Arr3b expressed in blue- and UV-sensitive cones, and carp cArr2 as zebrafish Arr3a expressed in red- and green-sensitive cones; Ref. 11). Their concentrations in the cytoplasm in the OS in the dark were estimated as follows: it was 0.24 mM in rods (total concentration of rArr1 plus rArr2), 0.6 mM in blue- and UV-sensitive cones, and 0.78 mM in red- and green-sensitive cones (Table 2C). These estimated concentrations are similar to those in mice reported previously (12, 35). Visual pigment concentration has been known to be 3 mM or so in the OS (18). In the estimation of the arrestin subtype concentration, we assumed that the cytoplasmic volume of the OS is half of the envelope volume (see “Results”) (31), so the molar ratio of arrestin to visual pigment in the OS envelope volume is 0.04 (0.12/3) in rods, 0.1 (0.3/3) in blue- and UV-sensitive cones, and 0.13 (0.39/3) in red- and green-sensitive cones. When light stimulus is given to rods and cones in the dark, maximally, rod arrestins, rArr1 and rArr2, can bind to 4% of the pigment; cone arrestin, cArr1 can bind 10% of the pigment, and cArr2 can bind 13% of the pigment. Above this bleaching level, arrestin distributing throughout rods and cones must be moved from the other part of the cell to the OS to exert its effect further. Because rods function in dim light, which causes negligible production of R*, the expression level of rod arrestins could be sufficient in their normal functional range of light.

We also quantified the amount of arrestins in whole part of a rod or a cone as the ratio to that of visual pigment. The ratio of the content of rArr1 plus rArr2 to that of pigment was 0.29 in rods, and the ratios in cones were 3.46 in blue/UV-sensitive cones (cArr1) and 3.22 in red/green-sensitive cones (cArr2) (Table 1). This quantification suggests that even if all of arrestin molecules move to the OS at the time when all pigment molecules are bleached in rods, only 30% of R* can bind to arrestins. In contrast, all type of cones contain excess amounts of cArr1 or cArr2 to bind even when all of the pigment is bleached. It is not known whether all of the arrestin molecules in a rod or a cone actually move to the OS in the light. In any case, the higher expression levels of cArr1 and cArr2 suggest very effective inactivation of R* in these cones no matter whether the pigment is phosphorylated or nonphosphorylated.

**Arrestin Binding to the Pigment in Rods—**Rod arrestin has been reported to bind preferentially to phosphorylated R* and not significantly to nonphosphorylated R* or dark state rhodopsin (23). In addition, PDE activation was suppressed by arrestin in the presence of visual pigment phosphorylation but not in its absence (5). Based on these previous findings in rods, it has been thought that arrestins bind to visual pigments only after R* is phosphorylated by GRK to completely suppress the activity of R*. However, this study clearly showed that rod (and also cone) arrestins suppress Tr* formation in the absence of visual pigment phosphorylation (Figs. 3 and 4) and also that rod arrestins, rArr1 and rArr2, actually bind to nonphosphorylated R* transiently (Fig. 5B). Therefore, our study strongly suggests the involvement of arrestin in quenching the activity of R* without pigment phosphorylation.

The reason for the failure to observe the binding of arrestin to nonphosphorylated pigment would be due to the transient binding of arrestin to R*. The reason why PDE activity was not affected by arrestin would be due to the low concentrations of arrestin used in the previous PDE activation study; as quantified in this study, concentration of arrestin, either rArr1 or rArr2, in the OS in the dark is ~100 μM in rods, although in the earlier study, where no effect of rod arrestin on PDE activation was observed in the absence of pigment phosphorylation, rod arrestin concentration was ~1.5 μM (5). At this concentration of rod arrestins, their effects without pigment phosphorylation would be small (Fig. 4, A and B).

In intact cells, R* is phosphorylated by GRK, and therefore, carp rod arrestins can bind to either phosphorylated R* or nonphosphorylated R*. From the result of firm binding to phosphorylated R*, rArr2 seems to correspond to rod arrestin in warm-blooded animals. In this case, it is not certain whether rod arrestin in warm-blooded animals binds to nonphosphorylated pigment in rods as carp rArr2 does. In the study in GRK1 and arrestin double knock-out mice, light response recovery was slower than that observed in GRK1 alone knock-out mice (14),...
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which supports our view that rod arrestin actually binds to non-phosphorylated R* to suppress Tr* formation. There could be an arrestin-binding site, which is not associated with pigment phosphorylation, in R*.

It has been suggested that there are two separate receptor-binding sites in rod arrestin, one recognizing phosphate(s) incorporated into R* and the other recognizing the R* conformation (28). The effect of ATP alone is due to the quench of R* by phosphorylation on R* with GRK, and the effect of arrestin alone could be due to binding of arrestin to nonphosphorylated R* through the R* recognition site(s). The suppression effect of ATP plus arrestin, which was higher than that of ATP or arrestin alone (Figs. 3 and 4), could be attributed to binding of arrestin to phosphorylated R* through the phosphate recognition site(s). Alternatively, this higher effect could be due to additive effects of R* phosphorylation and binding of arrestin to R* through the R* recognition site(s). In this study, it is not certain which case it was.

Several important amino acid residues are suggested to be present in these binding sites (28). Among them, the “finger loop,” the region possibly required for binding to R* (amino acid residues 68–78 in bovine arrestin-1), is mostly conserved in all of the carp arrestins (rArr1, rArr2, cArr1, and cArr2), which explains the binding of arrestins examined (rArr1, rArr2, and cArr2) to nonphosphorylated R* and phosphorylated R*. This loop is suggested to be exposed as a result of arrestin binding to the phosphate(s) in phosphorylated R* through the phosphate recognition site(s) (28). However, this loop may bind to nonphosphorylated R* directly and transiently in carp arrestins. The structural mechanism of transient binding observed in this study may be different from that suggested previously for arrestin to bind firmly to phosphorylated R*. Several sites in arrestin are proposed to be required for firm binding of arrestin to phosphorylated R* but not to nonphosphorylated R*: a C-terminal region (36) and, in addition, the four amino acid residues to detect phosphate(s) in phosphorylated R* (Lys-14, Lys-15, Arg-175, and Asp-296, in bovine arrestin-1) (28). All carp arrestins, including those examined (rArr1, rArr2, and cArr2), contain the above four residues, and rArr1 and rArr2 retain the C terminus (in cArr2, the C terminus is truncated). Therefore, we could predict that rArr1 and rArr2 bind firmly to phosphorylated R* but not to nonphosphorylated R*. However, both were found to bind transiently to nonphosphorylated R*, and moreover, rArr1 did not show firm binding to phosphorylated R* (Fig. 5B). Further study seems to be required to fully understand the functional mechanism of arrestin.

In Fig. 3, A and B, Tr* formation did not seem to be affected by ATP or rod arrestins in a certain time range after a light flash. It was 1 s in rods (see Fig. 3F, left panel, for rArr1). Within this time range, R* seems to allow the binding of only Tr not of other molecules such as GRK or arrestin, which may suggest that a certain transient state of a bleaching intermediate interacts only to Tr (see below). In GRK1 knock-out (3) and also in arrestin knock-out mice (6), the rising phase of a rod response has been shown to be unaffected by either of these proteins up to ~0.1 ms after a light flash. Although the time range is different between carp and mice, which is probably due to the difference in the temperature where the measurements were made, R* seems to react only to Tr just after a light flash in both animals.

The binding time course of rArr1 and of rArr2 was obviously different (Fig. 5B, top and middle panels). Binding of rArr1 to rod membranes was transient regardless of pigment phosphorylation, although the binding to nonphosphorylated R* was more transient. In contrast, rArr2 binds transiently to nonphosphorylated R* but firmly binds to phosphorylated R*. All of the binding difference could arise from the difference in the substrate specificity between rArr1 and rArr2. Based on our measurements of GTP§yS binding time course (Fig. 3, A and B) and rod arrestin binding time courses (Fig. 5B, top and middle panels), we speculate that there are at least four transient states of R* relating to the binding of rod arrestin. Here, we assume that they are R*1 to R*4 and that they are formed in this order in the bleaching process. Rod arrestins, rArr1 and rArr2, do not bind to R*1, which is formed within 1 s after a light flash and reacts only to Tr (Fig. 3, A and B, and left panel in F). On the conversion from R*1 to R*2 at ~1 s after a light flash, both rArr1 and rArr2 bind to R*2, some of which are phosphorylated but others may not be phosphorylated. Regardless of phosphorylation, both rod arrestins bind to R*2 to suppress the formation of Tr*. R*2 will decay to R*3 at ~1 min after a light flash (Fig. 5B, top and middle panels). Both rArr1 and rArr2 dissociate from nonphosphorylated R*3 (Fig. 5B, open circles), although both of them still remain bound to phosphorylated R*3 until it converts to R*4 at ~5 min after a light flash (filled circles). On this conversion, rArr1 dissociates from phosphorylated R*4, but rArr2 still remains bound to phosphorylated R*4. It has been shown that the half-life of metarhodopsin II in goldfish is ~5 min (37). Therefore, one of the transient states could be metarhodopsin II, but it is also possible that other transient states that could not be distinguished from metarhodopsin II spectrophotometrically cause the binding difference of rod arrestins in the presence and absence of pigment phosphorylation. Therefore, our results suggest dynamic conformational changes in the opsin moiety in physiologically important bleaching intermediates of visual pigment. The results also suggest that transient binding of arrestin is very effective in quenching the activity of R*, possibly through facilitation of the bleaching process.

Differently from warm-blooded animals, carp have two subtypes of arrestins in their rods, rArr1 and rArr2. One arrestin, rArr2, shows firm binding to phosphorylated R* like the arrestin in warm-blooded animals, and the other, rArr1, does not show the firm binding. Both show transient binding to nonphosphorylated R*. Because rArr1 and rArr2 are co-expressed in a rod in carp, R* will be inactivated with either of these arrestins in a time-dependent manner depending on R* phosphorylation by GRK1. Even in mice that have higher body temperatures than carp, a significant portion of R* is still phosphorylated at 10 min after bleaching (38, 39). Because phosphorylated R* still retains residual activity to form Tr* (5), firm binding of rArr2 to phosphorylated R* would contribute to quench the residual activity of phosphorylated R*. However, the amount of rod arrestins expressed in carp is less than that of the pigment (see above), so rod arrestins may exert their effects only under dim light.
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Arrestin Binding to the Pigment in Cones—In cones, cArr2 also suppressed Tr* formation in the absence of ATP (Figs. 3D and 4C). Although the binding of cArr2 was measured at a temperature lower than that used for the measurement of Tr* formation (4 °C versus room temperature), it is evident that cArr2 binds to cone R* transiently (Fig. 5B, bottom panel). Therefore, suppression of Tr* formation with cArr2 would be attained by this transient binding of cArr2 to phosphorylated or nonphosphorylated cone R*. In Nrl−/− mice, cone arrestin binds firmly to phosphorylated cone R* (40). One possible explanation for this apparent inconsistency between transient binding of carp cone arrestin and firm binding of mouse cone arrestin to phosphorylated cone R* would be that a structurally important C-terminal hydrophobic sequence, which is Ile-367–Val-368–Ile-369 in mouse cone arrestin (NM_133205.3), is truncated in carp cArr2.

As shown above, Tr* formation in rods did not seem to be affected by ATP or arrestins during ~1 s after a light flash (Fig. 3, A and B). In cones, Tr* formation seems to be affected by cArr2 alone at our earliest time point (125 ms, see Fig. 3F, right panel), which suggests that the lifetime of cone R*1 is shorter than 125 ms. In our previous study, ~1 phosphate was incorporated into cone R* at 125 ms after a light flash, which would be due to very effective GRK7 activities (15). Although we could not measure the cArr2 binding time course at room temperature, cArr2 probably binds to phosphorylated cone R*2 or subsequent transition states and dissociates from them later. All of these facilitated time courses of the effect of cArr2 would arise from shorter lifetimes of bleaching intermediates in cone pigments than in rod pigments (41). Although we did not study the binding of cArr1 expressed in blue/UV-sensitive cones, at least red/green-sensitive cones in carp do not express arrestins showing firm binding to phosphorylated cone R*. This is possibly because the lifetime of bleaching intermediate in cones is short enough, and suppression by binding to phosphorylated R* is not necessary.

At 30 μM arrestin concentration, the effect of cArr2 on the suppression of Tr* formation is higher than that of rArr1 or rArr2 (Fig. 3), which suggests higher binding affinity of cArr2 to nonphosphorylated R* in cones. Actually, the dose-response relation of cArr2 showed almost a maximum suppression effect at 3 μM concentration (Fig. 4C) in contrast to rod arrestins that caused a half-maximum effect at ~150 μM (rArr1) and ~8 μM (rArr2). As shown, cArr2 concentration in the cone OS in the dark is estimated to be ~0.8 mM, which is 26 times higher than the cArr2 concentration used in the study in Fig. 3 (30 μM), so that the suppression in intact cones could be much more effective, specifically in the speed of suppression, than that observed in this study.

In this study, we found direct suppression of nonphosphorylated R* by rod and cone arrestins. At physiological concentrations of arrestin in the OS (~0.25 mM in rods and 0.6–0.8 mM in cones), arrestin probably acts as a quencher of R* regardless of pigment phosphorylation. This role is overlapped with that of pigment phosphorylation and could be a dual mechanism of R* quench just after a light flash in both rods and cones. Although carp rods and cones express different subtypes of arrestins, their roles seem to be similar in a qualitative way but different in a quantitative way.

REFERENCES

1. Kawamura, S., and Tachibanaki, S. (2008) Rod and cone photoreceptors. Molecular basis of the difference in their physiology. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 150, 369–377
2. Fu, Y., and Yau, K. W. (2007) Phototransduction in mouse rods and cones. Pflugers Arch. 454, 805–819
3. Chen, C. K., Burns, M. E., Spencer, M., Niemi, G. A., Chen, J., Hurley, J. B., Baylor, D. A., and Simon, M. I. (1999) Abnormal photoresponses and light-induced apoptosis in rods lacking rhodopsin kinase. Proc. Natl. Acad. Sci. U.S.A. 96, 3718–3722
4. Kühn, H., Hall, S. W., and Wilden, U. (1984) Light-induced binding of 48-kDa protein to photoreceptor membranes is highly enhanced by phosphorylation of rhodopsin. FEBS Lett. 176, 473–478
5. Wilden, U., Hall, S. W., and Kühn, H. (1986) Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. Proc. Natl. Acad. Sci. U.S.A. 83, 1174–1178
6. Xu, J., Dodd, R. L., Makino, C. L., Simon, M. I., Baylor, D. A., and Chen, J. (1997) Prolonged photoresponses in transgenic mouse rods lacking arrestin. Nature 389, 505–509
7. Craft, C. M., Whitmore, D. H., and Wiechmann, A. F. (1994) Cone arrestin identified by targeting expression of a functional family. J. Biol. Chem. 269, 4613–4619
8. McKechnie, N. M., Al-Mahdawi, S., Dutton, G., and Forrester, J. V. (1986) Ultrastructural localization of retinal S antigen in the human retina. Exp. Eye. Res. 42, 479–487
9. Sakuma, H., Inana, G., Murakami, A., Higashide, T., and McLaren, M. J. (1996) Immunolocalization of X-arrestin in human cone photoreceptors. FEBS Lett. 382, 105–110
10. Smith, W. C., Gurevich, E. V., Dugger, D. R., Vishnivetskiy, S. A., Shelamer, C. L., McDowell, J. H., and Gurevich, V. V. (2000) Cloning and functional characterization of salmon rod and cone arrestins. Invest. Ophthalmol. Vis. Sci. 41, 2445–2455
11. Renninger, S. L., Gesemann, M., and Neuhauss, S. C. (2011) Cone arrestin confers cone vision of high temporal resolution in zebrafish larvae. Eur. J. Neurosci. 33, 658–667
12. Nikonov, S. S., Brown, B. M., Davis, J. A., Zuniغا, I. F., Bragin, A., Pugh, E. N., Jr., and Craft, C. M. (2008) Mouse cones require an arrestin for normal inactivation of phototransduction. Neuron 59, 462–474
13. Tachibanaki, S., Tsushima, S., and Kawamura, S. (2001) Low amplification and fast visual pigment phosphorylation as mechanisms characterizing cone photoreceptors. Proc. Natl. Acad. Sci. U.S.A. 98, 14044–14049
14. Burns, M. E., Mendez, A., Chen, C. K., Almuente, A., Quillinan, N., Simon, M. I., Baylor, D. A., and Chen, J. (2006) Deactivation of phosphorylated and nonphosphorylated rhodopsin by arrestin splice variants. J. Neurosci. 26, 1036–1044
15. Tachibanaki, S., Arinobu, D., Shimauchi-Matsukawa, Y., Tsushima, S., and Kawamura, S. (2005) Highly effective phosphorylation by G protein-coupled receptor kinase 7 of light-activated visual pigment in cones. Proc. Natl. Acad. Sci. U.S.A. 102, 9329–9334
16. Shimauchi-Matsukawa, Y., Aman, Y., Tachibanaki, S., and Kawamura, S. (2005) Isolation and characterization of visual pigment kinase-related genes in carp retina: polyphyly in GRK1 subtypes, GRK1A and 1B. Mol. Vis. 11, 1220–1228
17. Gurevich, V. V., and Benovic, J. L. (2000) Arrestin: mutagenesis, expression, purification, and functional characterization. Methods Enzymol. 315, 422–437
18. Hárosi, F. I. (1975) Absorption spectra and linear dichroism of some amphibian photoreceptors. J. Gen. Physiol. 66, 357–382
19. Arinobu, D., Tachibanaki, S., and Kawamura, S. (2010) Larger inhibition of visual pigment kinase in cones than in rods. J. Neurochem. 115, 259–268
20. Tachibanaki, S., Yonetsu, S., Fukaya, S., Koshitani, Y., and Kawamura, S. (2012) Low activation and fast inactivation of transducin in carp cones. J. Biol. Chem. 287, 41186–41194
21. Koshitani, Y., Tachibanaki, S., and Kawamura, S. (2014) Quantitative aspects of cGMP phosphodiesterase activation in carp rods and cones. *J. Biol. Chem.* 289, 2651–2657

22. Kimura, N., and Shimada, N. (1983) GDP does not mediate but rather inhibits hormonal signal to adenylate cyclase. *J. Biol. Chem.* 258, 2278–2283

23. Gurevich, V. V., and Benovic, J. L. (1992) Cell-free expression of visual arrestin. Truncation mutagenesis identifies multiple domains involved in rhodopsin interaction. *J. Biol. Chem.* 267, 21919–21923

24. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917

25. Morrison, W. R. (1964) A fast, simple, and reliable method for the micro-determination of phosphorus in biological materials. *Anal. Biochem.* 7, 218–224

26. Shimauchi-Matsukawa, Y., Aman, Y., Tachibanaki, S., and Kawamura, S. (2008) Identification of differentially expressed genes in carp rods and cones. *Mol. Vis.* 14, 358–369

27. Imanishi, Y., Hitomi, O., and Tokunaga, F. (1999) Two types of arrestins expressed in medaka rod photoreceptors. *FEBS Lett.* 462, 31–36

28. Gurevich, V. V., Hanson, S. M., Song, X., Vishnivetskiy, S. A., and Gurevich, E. V. (2011) The functional cycle of visual arrestins in photoreceptor cells. *Prog. Retin. Eye Res.* 30, 405–430

29. Zhang, H., Cueca, N., Ivanova, T., Church-Kopish, J., Frederick, J. M., MacLeish, P. R., and Baehr, W. (2003) Identification and light-dependent translocation of a cone-specific antigen, cone arrestin, recognized by monoclonal antibody 7G6. *Invest. Ophthalmol. Vis. Sci.* 44, 2858–2867

30. Zhu, X., Li, A., Brown, B., Weiss, E. R., Osawa, S., and Craft, C. M. (2002) Mouse cone arrestin expression pattern: light induced translocation in cone photoreceptors. *Mol. Vis.* 8, 462–471

31. Fein, A., and Szuts, E. Z. (1982) in *Photoreceptors: Their Roles in Vision* (Hutchinson, F., Fuller, W., Mullins, L. J., and Villegas, R., eds) p. 33, Cambridge University Press, Cambridge, UK

32. Kennedy, M. J., Dunn, F. A., and Hurley, J. B. (2004) Visual pigment phosphorylation but not transducin translocation can contribute to light adaptation in zebrafish cones. *Neuron* 41, 915–928

33. Sutton, R. B., Vishnivetskiy, S. A., Robert, J., Hanson, S. M., Raman, D., Knox, B. E., Kono, M., Navarro, J., and Gurevich, V. V. (2005) Crystal structure of cone arrestin at 2.3 Å: Evolution of receptor specificity. *J. Mol. Biol.* 354, 1069–1080

34. Luo, D. G., Yue, W. W., Ala-Laurila, P., and Yau, K. W. (2011) Activation of visual pigments by light and heat. *Science* 332, 1307–1312

35. Song, X., Vishnivetskiy, S. A., Seo, J., Chen, J., Gurevich, E. V., and Gurevich, V. V. (2011) Arrestin-1 expression level in rods: balancing functional performance and photoreceptor health. *Neuroscience* 174, 37–49

36. Pulvermüller, A., Maretski, D., Rudnicka-Nawrot, M., Smith, W. C., Palczewski, K., and Hofmann K. P. (1997) Functional differences in the interaction of arrestin and its splice variant, p44, with rhodopsin. *Biochemistry* 36, 9253–9260

37. Golobokova, E. Y., and Govardovskii, V. I. (2006) Late stages of visual pigment photolysis in situ. Cones vs. rods. *Vision Res.* 46, 2287–2297

38. Ohguro, H., Van Hooser, J. P., Milam, A. H., and Palczewski, K. (1995) Rhodopsin phosphorylation and dephosphorylation in vivo. *J. Biol. Chem.* 270, 14259–14262

39. Kennedy, M. J., Lee, K. A., Niemi, G. A., Craven, K. B., Garwin, G. G., Saari, J. C., and Hurley, J. B. (2001) Multiple phosphorylation of rhodopsin and the in vivo chemistry underlying rod photoreceptor dark adaptation. *Neuron* 31, 87–101

40. Zhu, X., Brown, B., Li, A., Mears, A. J., Swaroop, A., and Craft, C. M. (2003) GRK1-dependent phosphorylation of S and M opsins and their binding to cone arrestin during cone phototransduction in the mouse retina. *J. Neurosci.* 23, 6152–6160

41. Shichida, Y., and Imai, H. (1998) Visual pigment: G-protein-coupled receptor for light signals. *Cell. Mol. Life Sci.* 54, 1299–1315