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ORIGINAL ARTICLE

# Binding of rhodopsin and rhodopsin analogues to transducin, rhodopsin kinase and arrestin-1

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## Abstract

**AIM:** To investigate the interaction of reconstituted rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin with transducin, rhodopsin kinase and arrestin-1.

**METHODS:** Rod outer segments (ROS) were isolated from bovine retinas. Following bleaching of ROS membranes with hydroxylamine, rhodopsin and rhodopsin analogues were generated with the different retinal isomers and the concentration of the reconstituted pigments was calculated from their UV/visible absorption spectra. Transducin and arrestin-1 were purified to homogeneity by column chromatography, and an enriched-fraction of rhodopsin kinase was obtained by extracting freshly prepared ROS in the dark. The guanine nucleotide binding activity of transducin was determined by Millipore filtration using β,γ-imido-($^{3}$H)-guanosine 5′-triphosphate. Recognition of the reconstituted pigments by rhodopsin kinase was determined by autoradiography following incubation of ROS membranes containing the various regenerated pigments with partially purified rhodopsin kinase in the presence of (γ-$^{32}$P) ATP. Binding of arrestin-1 to the various pigments in ROS membranes was determined by a sedimentation assay analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

**RESULTS:** Reconstituted rhodopsin and rhodopsin analogues containing 9-*cis*-retinal and 13-*cis*-retinal rendered an absorption spectrum showing a maximum peak at 498 nm, 486 nm and about 467 nm, respectively, in the dark; which was shifted to 380 nm, 404 nm and about 425 nm, respectively, after illumination. The percentage of reconstitution of rhodopsin and the rhodopsin analogues containing 9-*cis*-retinal and 13-*cis*-retinal was estimated to be 88%, 81% and 24%, respectively. Although only residual activation of transducin was observed in the dark when reconstituted rhodopsin and 9-*cis*-retinal-rhodopsin was used, the rhodopsin analogue containing the 13-*cis* isomer of retinal was capable of activating transducin independently of light. Moreover, only a basal amount of the reconstituted rhodopsin and 9-*cis*-retinal-rhodopsin was phosphorylated by rhodopsin kinase in the dark, whereas the pigment containing the 13-*cis*-retinal was highly phosphorylated by rhodopsin kinase even in the dark. In addition, arrestin-1 was incubated with rhodopsin, 9-*cis*-retinal-rhodopsin or 13-*cis*-retinal-rhodopsin. Experiments were performed using both phosphorylated and non-phosphorylated regenerated pigments. Basal amounts of arrestin-1 interacted with rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin under dark and light conditions. Residual arrestin-1 was also recognized by the phosphorylated rhodopsin and phosphorylated 9-*cis*-retinal-rhodopsin in the dark. However, arrestin-1 was recognized by phosphorylated 13-*cis*-

retinal-rhodopsin in the dark. As expected, all reformed pigments were capable of activating transducin and being phosphorylated by rhodopsin kinase in a light-dependent manner. Additionally, all reconstituted photolyzed and phosphorylated pigments were capable of interacting with arrestin-1.

**CONCLUSION:** In the dark, the rhodopsin analogue containing the 13-*cis* isomer of retinal appears to fold in a pseudo-active conformation that mimics the active photointermediate of rhodopsin.



**Key words:** Rhodopsin; Rhodopsin analogues; 9-*cis*-Retinal; 11-*cis*-Retinal; 13-*cis*-Retinal; Photointermediates; Transducin; Rhodopsin kinase; Arrestin-1; Visual process

**Core tip:** Rhodopsin is a specialized G protein-coupled receptors composed of a single polypeptide chain, opsin, and a covalently linked 11-*cis*-retinal. It is well known that rhodopsin uses the 11-*cis* form of retinal exclusively as the chromophore. Retinal analogues have long been used to probe the chromophore binding pocket and to study ligand-protein relationships to better understand the photochemical cis-trans isomerization of rhodopsin. However, little is known about the interactions of rhodopsin analogues with other proteins in the visual cascade. Here, we were able to reconstitute a rhodopsin analogue containing 13-*cis*-retinal. We compared the binding of reconstituted rhodopsin, 9-cis-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin to transducin, rhodopsin kinase and arrestin-1, both in the dark and under illumination. Interestingly, we found that in the dark the rhodopsin analogue containing the 13-*cis* isomer of retinal appears to fold in a pseudo-active conformation that mimics the active photointermediate of rhodopsin.

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## INTRODUCTION

G protein-coupled receptors (GPCRs) activate signaling paths in response to a diverse number of stimuli such as photons, $Ca^{2+}$, organic odorants, amines, hormones, nucleotides, nucleosides, peptides, lipids and even large proteins[1]. All GPCRs share a conserved seven-transmembrane-helix structural bundle connected by six loops of varying lengths. Binding of specific ligands to the transmembrane or extracellular domains of members of the GPCR superfamily causes conformational changes that act as a switch to relay the signal to heterotrimeric G proteins that in turn evoke further intracellular responses[2].

The dim-light photoreceptor rhodopsin is a highly specialized GPCR composed of a single polypeptide chain of 348 amino acids that conforms the apoprotein opsin, and a covalently linked 11-*cis*-retinal chromophore that is tightly packed within the bundle of helices[3,4]. The chromophore is bound to the ε-amino group of Lys296, located in the seventh helix (TM7) *via* a protonated Schiff base linkage. In the ground state this charge is stabilized by the counter-ion Glu113 that is located in the third helix (TM3)[5]. Another important structural feature of the 11-*cis*-retinal chromophore in rhodopsin is its extended polyene structure, which accounts for its visible absorption properties and allows for resonance structures[6].

In rhodopsin, 11-*cis*-retinal serves both as the chromophore and as an inverse agonist that holds the visual pigment protein in an inactive conformation. Absorption of a photon by the 11-*cis*-retinal of rhodopsin causes its photoisomerization to the all-*trans* form[7], converting the ligand into an agonist, and leading to a conformational change of the protein moiety that triggers the signal transduction cascade *via* reactions of the G protein transducin. Following *cis-trans* isomerization of the chromophore, rhodopsin relaxes through a series of photoproducts, which have been identified by their characteristic absorption spectra. One of the photointermediates, metarhodopsin Ⅱ (meta Ⅱ), is the active conformation of rhodopsin responsible of binding transducin and initiating the signaling process. Transducin, which is arranged as two units, the α subunit and the βγ-complex, transmits the visual stimuli by activating a potent cGMP phosphodiesterase known as PDE6. The resulting decrease in the cytosolic concentration of cGMP causes the closure of cation-specific cGMP-gated channels located in the plasma membrane, leading to the hyperpolarization of the rod cell. Additional protein molecules participate in modulating the duration of the signal and the achievement of the appropriate response[8]. Particularly, the phosphorylation of photoactivated rhodopsin by rhodopsin kinase, also known as GPCR kinase 1 or GRK1, and its interaction with arrestin-1, are both involved in signal desensitization since the transducin activation phase is terminated by the interaction of meta Ⅱ with rhodopsin kinase and arrestin-1[9,10]. Subsequently, the retinal Schiff base is hydrolysed and the photolysed all-*trans*-retinal is released from its binding site. Regeneration of the light sensitive rhodopsin ground state requires the supply of new 11-*cis*-retinal through the so-called retinoid cycle[11,12].

It is well known that the rod visual pigment rhodopsin uses the 11-*cis* form of retinal exclusively as the chromophore, and the strict selection of this isomer appears to have occurred early in the evolution of visual function. Under certain pathological conditions, however, also the 9-*cis* configuration of retinal is observed, which generates a pigment known as isorhodopsin[13]. Retinal analogues have long been used to probe the chromophore binding pocket and to study ligand-protein relationships to better understand the photochemical *cis-trans* isomerization of rhodopsin[14] and cone opsins[15]. Yet, little is known about the interac-

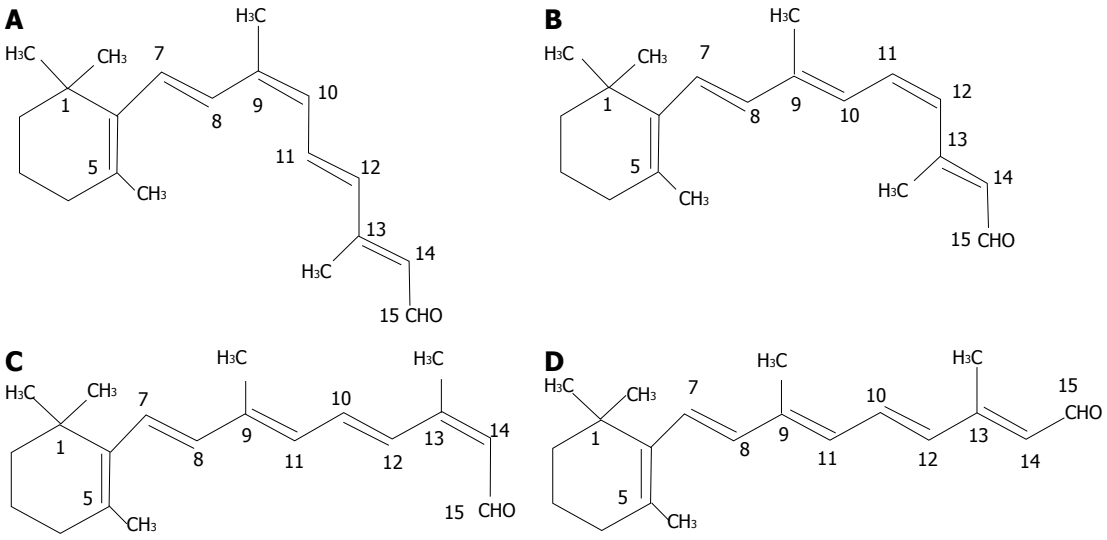


**Figure 1 Structures of retinal analogues.** A: 9-*cis*-retinal; B: 11-*cis*-retinal; C: 13-*cis*-retinal; D: all-*trans*-retinal.

tions of rhodopsin analogues with other proteins in the visual cascade. Although, it has been reported that the retinal binding site in the inactive state of rhodopsin can accommodate the 7-*cis*, 9-*cis* and 11-*cis* isomers of retinal, but not the longer all-*trans* or 13-*cis* isomers[16], in the present work we were able to reconstitute a rhodopsin analogue containing 13-*cis*-retinal, in addition to photoreceptor proteins containing 9-*cis*-retinal and 11-*cis*-retinal. We compared the binding of reconstituted rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin to transducin, rhodopsin kinase and arrestin-1, both in the dark and under illumination. The chemical structures of the geometrical retinal isomers used here and of all-*trans*-retinal are shown in Figure 1.

## MATERIALS AND METHODS

### Materials

Bovine eyes were obtained from the nearest abattoir (Beneficiadora Diagon, CA, Matadero Caracas, Venezuela). Retinae were extracted in the dark, under red light, and were maintained frozen at -80 ℃. Reagents were purchased from the following sources: β,γ-imido-($^3$H)-guanosine 5'-triphosphate [(8-$^3$H) GMPpNp] (17.9 Ci/mmol) and (γ-$^{32}$P) ATP (3000 Ci/mmol), Amersham; 9-*cis*-retinal, 13-*cis*-retinal, bovine serum albumine (BSA), hydroxylamine, phytic acid or inositol hexakisphosphate ($IP_6$), n-dodecyl β-D-maltoside, and DEAE-cellulose, Sigma-Aldrich; ATP, heparine-sepharose and concanavalin A-Sepharose 4B, Pharmacia; molecular weight pre-stained protein markers, and Bradford reagent, Bio-Rad; anti-rabbit IgG antibodies conjugated to alkaline phsophatase, KPL; bromocloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT), and molecular weight protein standards, Promega; X-ray films, Kodak. The 11-*cis*-retinal was donated by Dr. Debra Thompson, University of Michigan, United States.

### Preparation of rod outer segments and washed membranes

Rod outer segments (ROS) were isolated from frozen bovine retinas as described previously[17]. Dark depleted ROS membranes were prepared by washing ROS with 5 mmol/L Tris-HCl (pH = 7.4), 2 mmol/L EDTA, and 5 mmol/L β-mercaptoethanol until no significant amount of peripheral proteins was released with the wash buffer. ROS and dark-depleted ROS membranes were stored in the dark at -80 ℃. Rhodopsin concentration was calculated from its UV/visible absorption spectra, using its molar extinction coefficient (40700 $M^{-1}cm^{-1}$, at 500 nm)[18]. In addition, rhodopsin was identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using anti-bovine rhodopsin polyclonal antibodies raised in mice.

### Purification of transducin

Transducin was obtained from ROS prepared under room light, at 4 ℃, following the affinity procedure described by Kühn[19]. GTP (100 μmol/L) was employed to elute transducin from the washed illuminated ROS, and transducin was further purified to homogeneity by anion exchange chromatography on a DEAE-cellulose column as described elsewhere[20]. Fractions containing transducin were identified by SDS-PAGE and Western blot using anti-bovine transducin polyclonal antibodies raised in mice.

### Preparation of an Enriched fraction of Rhodopsin Kinase

Freshly prepared ROS were washed three times with an isotonic buffer containing 70 mmol/L potassium phosphate (pH = 6.8), 5 mmol/L magnesium acetate, 5 mmol/L β-mercaptoethanol, and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Following centrifugation, the washed ROS pellet was hypotonically extracted with 5 mmol/L Tris-HCl (pH = 7.4), 5 mmol/L magnesium acetate, 5 mmol/L β-mercaptoethanol, and 0.1 mmol/L PMSF[21]. Under these conditions, soluble proteins and proteins weakly associated with the membrane, including transducin, cGMP phosphodiesterase PDE6, arrestin-1, and rhodopsin kinase, appear in the supernatant gener-

ated after centrifugation. This supernatant was considered as the enriched fraction of rhodopsin kinase. The whole procedure was carried out at 4 ℃, in the dark under red light.

### Purification of arrestin-1

Arrestin-1 was purified following the procedure described by Buczylko *et al*[22]. Frozen bovine retinas were homogenized with 10 mmol/L Hepes (pH = 7.5), 0.1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol, under dim red light, at 4 ℃. Following centrifugation at 70000 *g*, for 25 min, the supernatant containing the soluble proteins was chromatographed on a DEAE-cellulose column, previously equilibrated in the same buffer. The column was washed with 10 mmol/L Hepes (pH = 7.5), 15 mmol/L NaCl, 0.1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol (Buffer A) until the absorbance at 280 nm dropped below 0.1. Adsorbed proteins were eluted with a 0 to 150 mmol/L linear gradient of NaCl in Buffer A, and the fractions containing arrestin-1 were identified by SDS-PAGE and Western blot using anti-bovine arrestin-1 polyclonal antibodies prepared in rabbits. These fractions were pooled and applied to a heparin-sepharose column, which was previously equilibrated with 10 mmol/L Hepes (pH = 7.5), 100 mmol/L NaCl, 0.1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol (Buffer B). Arrestin-1 was eluted using a gradient of 0 to 8 mmol/L phytic acid in Buffer B. The peak of arrestin-1 was pooled, dialyzed against Buffer A, applied to a second heparin-sepharose column, and eluted with 10 mmol/L Hepes (pH = 7.5), 400 mmol/L NaCl, 0,1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol.

### Bleaching of rhodopsin in washed ROS membranes

Washed ROS membranes were incubated with 50 mmol/L hydroxylamine in 10 mmol/L Tris-HCl (pH = 7.4), at 4 ℃, for 15 min, under illumination with a tungsten 100 W lamp. Then, the mixture was centrifuged at 50000 *g* for 20 min, at 4 ℃. The supernatant was discarded and the pellet was washed twice with 5 mmol/L Tris-HCl (pH = 7.4), 5 mmol/L magnesium acetate, 5 mmol/L β-mercaptoethanol.

### Regeneration of rhodopsin and rhodopsin analogues

Samples of bleached washed ROS membranes containing about 25 μmol/L of opsin were resuspended in 10 mmol/L Tris-HCl (pH = 7.4). Then, appropriate aliquots of stock solutions of 9-*cis*-retinal, 11-*cis*-retinal, and 13-*cis*-retinal prepared in ethanol were added in the dark. A molar ratio of 3:1 retinal to opsin was used for the reconstitution of the pigment with the 9-*cis*-retinal and 11-*cis*-retinal isomers, whereas a ratio of 15:1 retinal to opsin was employed for the regeneration of the rhodopsin analogue containing the 13-*cis*-retinal isomer. Following an overnight incubation, at room temperature, all samples were centrifuged at 50000 *g*, for 20 min, at 4 ℃. The regeneration of the pigments was followed by UV-Vis spectroscopy using the extinction coefficient of rhodopsin[18]. The excess of 9-*cis*-retinal, 11-*cis*-retinal, and 13-*cis*-retinal was eliminated by washing the membranes containing the reconstituted pigments with 2% BSA in 10 mmol/L Tris-HCl (pH = 7.4). BSA was then removed by successive washes with 5 mmol/L Tris-HCl (pH 7.4), 5 mmol/L magnesium acetate, 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol. ROS membranes containing the reconstituted pigments were resuspended in 5 mmol/L Tris-HCl (pH = 7.4), 100 mmol/L NaCl, 1 mmol/L magnesium acetate, 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol.

### Binding of (8-$^3$H) GMPpNp to transducin

Guanine nucleotide binding was measured by Millipore filtration using (8-$^3$H) GMPpNp, a radioactive non-hydrolyzable analogue of GTP, as previously described[23].

### Phosphorylation of reconstituted rhodopsin and rhodopsin analogues

ROS membranes containing the reconstituted pigments were incubated with a 50-μL aliquot of an enriched fraction of rhodopsin kinase, in the presence of 50 mmol/L Tris-HCl (pH = 7.5), 12 mmol/L $MgCl_2$, 20 mmol/L KF, 40 μmol/L [γ-$^{32}$P] ATP (specific activity about 4500 cpm/pmol), 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol. Following incubation for 1 h, at room temperature, under illumination with a 100 W tungsten lamp, the phosphorylated membranes were centrifuged at 100000 *g*, for 20 min, at 4 ℃. Identical control experiments were carried out in the dark. Samples were separated by SDS-PAGE and the phosphorylated bands were identified by autoradiography following staining and drying of the gels.

### Regeneration of phosphorylated rhodopsin and phosphorylated rhodopsin analogues

ROS containing 1.9 mg of rhodopsin were sedimented by centrifugation at 100000 *g* for 20 min, and resuspended in 50 mmol/L Tris-HCl (pH = 7.5), 12 mmol/L $MgCl_2$, 20 mmol/L KF, 40 μmol/L ATP, 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol, in the presence of a 50-μL aliquot of an enriched fraction of rhodopsin kinase. Following illumination for 1 h with a tungsten 100 W lamp, the mixture was centrifuged and the resulting pellet containing the phosphorylated protein was resuspended in 10 mmol/L Tris-HCl (pH = 7.4). Phosphorylated rhodopsin was bleached with 50 mmol/L hydroxylamine to obtain phosphorylated opsin. Samples of phosphorylated opsin were reconstituted with 9-*cis*-retinal, 11-*cis*-retinal and 13-*cis*-retinal as described above.

### Interaction of reconstituted rhodopsin and rhodopsin analogues with arrestin-1

The binding of arrestin-1 to the pigments reconstituted in washed ROS membranes was determined according to Gurevich *et al*[24], with slight modifications. Briefly, samples of arrestin-1 (14 μg) were incubated with 12 μg of the regenerated pigments, for 1 h, at room temperature. Experiments were performed in 100 μL of 5 mmol/L Tris-HCl (pH = 7.4), 5 mmol/L magnesium acetate, 0.1

mmol/L PMSF, 5 mmol/L β-mercaptoethanol, both in the dark and under illumination, using phosphorylated and non-phosphorylated pigments (about molar ratio of 1:1 arrestin-1 to pigment). The original mixture, and the resulting supernatant and pellet after centrifugation at 100000 *g*, for 20 min, were separated by SDS-PAGE. The gels were colored by silver staining and the bands of arrestin-1 and rhodopsin or rhodopsin analogues were evaluated by densitometry.

### Other procedures

Protein concentration was determined as reported by Bradford[25] using BSA as protein standard. SDS-PAGE was carried out on 1.5-mm thick slab gels containing 12% polyacrylamide as described by Laemmli[26]. Coomassie blue R-250 or silver staining was used for protein visualization. For Western blot analyses, the proteins were electrophoretically transferred from the gels to nitrocellulose sheets (0.45 μm pore size) as reported by Towbin *et al*[27]. Rhodopsin was purified to homogeneity by batchwise affinity chromatography on concanavalin A-Sepharose[28], using n-dodecyl β-D-maltoside instead of n-octyl β-D-glucopyranoside as the detergent. Polyclonal antibodies against rhodopsin and transducin were prepared in mice as described[29]. Purified arrestin-1 was used to raise polyclonal antibodies in rabbit serum following the procedure described by Harlow *et al*[30].

### Statistical analysis

For statistical analysis, mean value comparisons were performed by using the Student *t*-test or Anova and Krunskal-Wallis test. *P*-values below 0.05 were considered significant. Data in all histograms are graphed as mean ± SD.

## RESULTS

Analysis by SDS-PAGE showed that isolated ROS membranes contained all the proteins involved in the photoexcitation process (Figure 2A), including rhodopsin, transducin, cGMP phosphodiesterase PDE6, arrestin-1 and rhodopsin kinase[19]. As revealed by Western blot using anti-rhodopsin polyclonal antibodies, the major polypeptide band with an apparent molecular mass of approximately 35 kDa corresponded to rhodopsin (Figure 2B). Since rhodopsin has a tendency to oligomerize, higher order oligomers of rhodopsin, such as dimers, trimers, *etc*., were also detected by immunoblotting (Figure 2B). Rhodopsin polypeptide bands were observed in the original ROS sample and remained in the pellet following the washing procedure (Figure 2A and B). The presence of rhodopsin was also demonstrated by measuring the UV/visible absorption spectra of the samples and estimating the ratio of the absorbance at 280 nm to the absorbance at 500 nm[28,31]. Crude ROS showed a spectral ratio $A_{280\ nm}/A_{500\ nm}$ of 2.68, which decreased to 2.05 in dark-depleted ROS membranes after removal of the peripheral proteins (Figure 2C).

Rhodopsin was bleached by exposing washed ROS membranes to light in the presence of hydroxylamine. This treatment caused the complete detachment of the retinal chromophore. Rhodopsin and rhodopsin analogues containing the 9-*cis* and 13-*cis* isomers of retinal were reformed by incubating opsin with an excess of each retinal in the dark. The regeneration of rhodopsin is shown in Figure 2D as an example. As illustrated in Figure 2D, the 11-*cis*-retinal molecule possessed a broad absorption band at about 370 nm that overlapped with the absorption peak of the reconstituted rhodopsin pigment. Washes in the presence of BSA completely removed the residual retinal (Figure 2D).

As can be seen in Figure 3, pigments were reconstituted after the addition of the three retinal isomers to opsin in the dark. Reconstituted rhodopsin rendered the characteristic absorption spectrum of rhodopsin in the dark (Figure 3B), showing a maximum peak at 498 nm (about 500 nm). Following illumination, this band was shifted to 380 nm that corresponded to the meta Ⅱ photointermediate. In the dark, the reconstituted pigment analogue containing 9-*cis*-retinal (isorhodopsin) showed an absorbance peak at 486 nm (Figure 3A), which was slightly blue shifted in comparison to rhodopsin. Once photolyzed, the maximum of illuminated isorhodopsin was obtained at 404 nm which was slightly red shifted in comparison to meta Ⅱ. The absorption spectra of the rhodopsin analogue containing 13-*cis*-retinal showed broader bands than rhodopsin and isorhodopsin, under both, dark and light conditions (Figure 3C). In the dark, the absorption peak of 13-*cis*-retinal-rhodopsin was blue shifted showing its maximum at about 467 nm. After photolysis, the highest absorption peak of the illuminated 13-*cis*-retinal-rhodopsin was acquired at about 425 nm, more red shifted than meta Ⅱ and illuminated isorhodopsin. The percentage of reconstitution of the three pigments was estimated by comparing the absorption values at their maximum wavelength, using the extinction coefficient of rhodopsin as an approximate value[18], and the amount of total protein determined for each sample by the method of Bradford[25]. Our results showed that rhodopsin and isorhodopsin were reconstituted with a yield of 88% and 81%, respectively, whereas the rhodopsin analogue containing the 13-*cis* isomer of retinal was reformed with a yield of only 24%.

A partially purified transducin sample was initially obtained by GTP elution from illuminated ROS membranes. Then, transducin was purified to homogeneity by chromatography on a DEAE-cellulose column (Figure 4A). The elution of transducin was evaluated by measuring the rhodopsin- and light-dependent guanine nucleotide binding by a filtration assay using (8-$^3$H) GMPpNp. SDS-PAGE revealed that the same fractions comprising the GMPpNp binding activity also contained the polypeptide bands corresponding to the α-, β-, and γ-subunits of transducin (Figure 4A, Inset, top). In addition, anti-transducin polyclonal antibodies that preferentially detect

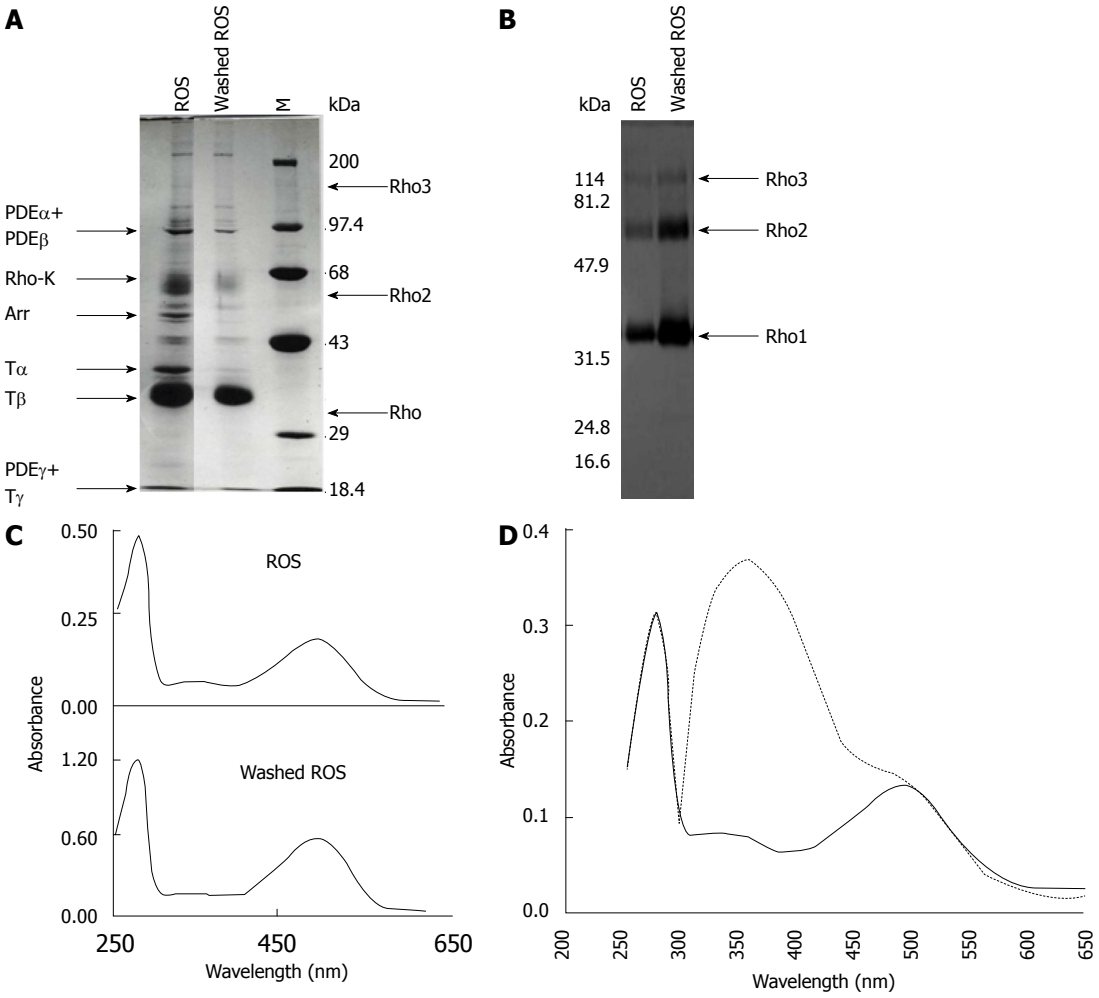


**Figure 2 Isolation of rod outer segments, preparation of washed rod outer segments membranes, and reconstitution of rhodopsin.** A: ROS were isolated from frozen bovine retinas and were hypotonically washed in the dark until no peripheral proteins were released. Arrows indicate the migration of rhodopsin (Rho), rhodopsin oligomers (Rho2 and Rho3), α-, β- and γ-subunits of the cGMP phosphodiesterase PDE6 (PDEα, PDEβ and PDEγ), α-, β- and γ-subunits of transducin (Tα, Tβ and Tγ), rhodopsin kinase (Rho-K), and arrestin-1 (Arr); B: ROS and dark-depleted ROS membranes were separated by SDS-PAGE, electrotransferred to a nitrocellulose filter and analyzed using polyclonal anti-rhodopsin antibodies. Arrows point out the migration of rhodopsin (Rho), rhodopsin dimers (Rho2), and rhodopsin trimers (Rho3). C: Absorption spectra of solubilized ROS and washed-ROS membranes in the dark; D: Regeneration of rhodopsin. A sample of depleted ROS membranes was bleached with hydroxylamine and incubated with an excess of 11-cis-retinal. Shown is the UV/visible spectra of rhodopsin in the dark, before (dashed line) and after (continuous line) removing the excess of 11-*cis*-retinal by washing with BSA. M: Molecular weight markers; ROS: Rod outer segments.

the α-subunit of transducin also recognized the α-subunit in the fractions containing the protein peak (Figure 4A, Inset, bottom).

Transducin binding to reconstituted rhodopsin and rhodopsin analogues was evaluated by measuring their capacity to induce the exchange of guanine nucleotides on transducin. The amount of reconstituted pigment, instead of the total amount of protein, was employed to normalize the reported values. As shown in Figure 5, all reformed pigments were capable of catalyzing the GMPpNp binding activity of transducin in a light-dependent manner. As expected, little activation of transducin (about 10%-15%) was observed in the dark when reconstituted rhodopsin and isorhodopsin were employed (Figure 5). Moreover, the apoprotein opsin was unable of inducing the exchange of GMPpNp on transducin (data not shown). In contrast and surprisingly, the rhodopsin analogue containing the 13-*cis* isomer of retinal was capable of activating transducin independently of light (about 40%) (Figure 5), suggesting that this pigment possesses a conformation in the dark that is similar to that of meta Ⅱ.

Figure 4B (left) shows the polypeptide composition of an aliquot of the enriched fraction of rhodopsin kinase, compared with samples of ROS and washed ROS membranes. This partially purified fraction of rhodopsin kinase contained polypeptide bands that corresponded to reported ROS peripheral proteins (transducin, cGMP phosphodiesterase PDE6, arrestin-1, rhodopsin kinase, *etc*.). As shown in Figure 4B (right) by autoradiography, intact ROS included active rhodopsin kinase given that rhodopsin was specifically phosphorylated in a light-dependent manner. Phosphorylated rhodopsin oligomers were also obtained in the crude ROS sample (Figure 4B, right). The enriched fraction of rhodopsin kinase was also capable of phosphorylating rhodopsin in washed-ROS membranes and under illumination (Figure 4B,

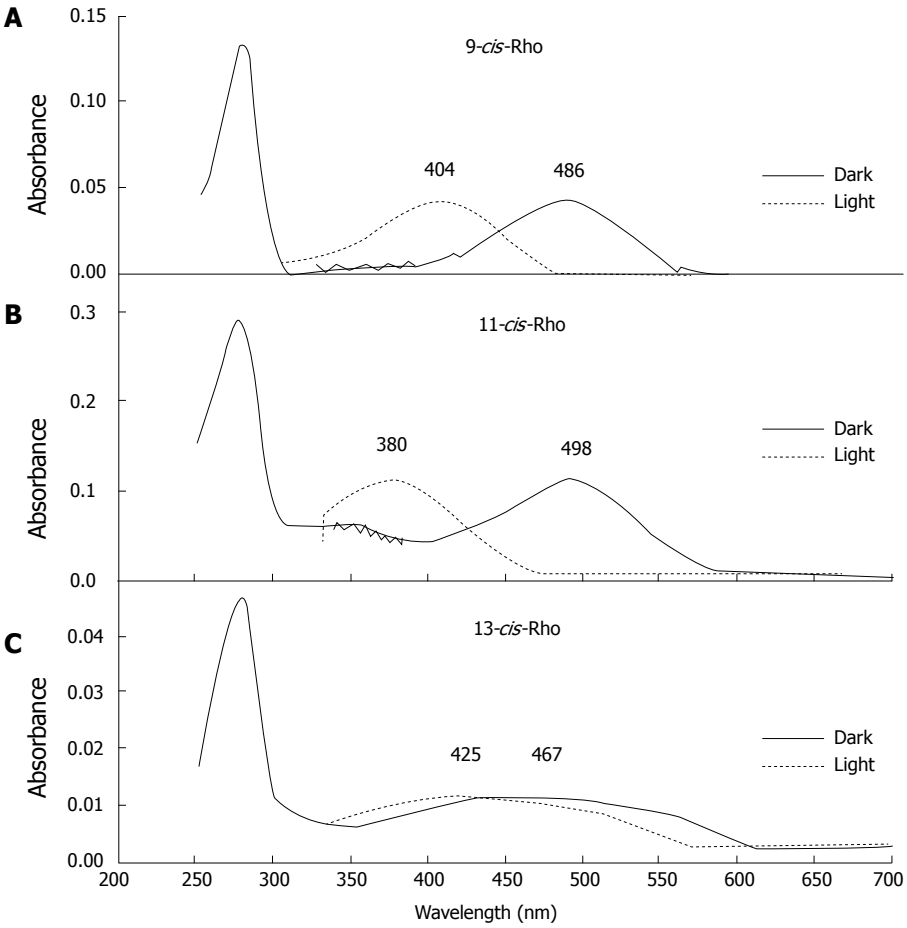


**Figure 3 Absorption spectra of rhodopsin and rhodopsin analogues.** Absorption spectrum of 9-*cis*-retinal-rhodopsin (9-*cis*-Rho) (A), rhodopsin (11-*cis*-Rho) (B) and 13-*cis*-retinal-rhodopsin (13-*cis*-Rho) (C) in the dark (continuous line) and under illumination (dashed line). Shown are the maximum wavelengths for each pigment.

right).

The ability of the reconstituted rhodopsin and rhodopsin analogues to serve as substrates for rhodopsin kinase was then measured by incubating each sample with an aliquot of the enriched fraction of rhodopsin kinase. As shown in Figure 6A by Coomassie blue staining, the same amount of each reconstituted protein was loaded in the gel lanes. Figure 6B illustrates by autoradiography that an enriched fraction of rhodopsin kinase was capable of phosphorylating all the reformed pigments in a light-dependent manner. Only basal amounts of the reconstituted rhodopsin and isorhodopsin samples were phosphorylated by rhodopsin kinase in the dark (Figure 6B). Opsin behaved similar to inactive rhodopsin given that the apoprotein was not phosphorylated by rhodopsin kinase (data not shown). However, the pigment containing the 13-*cis*-retinal was highly phosphorylated by rhodopsin kinase even in the dark (Figure 6B). Autoradiograms were quantified by densitometry in Figure 6C, corroborating the results qualitatively obtained in Figure 6B. The amount of regenerated pigment, instead of the total amount of protein, was used to normalize the reported values. These results suggest that 13-*cis*-retinal-rhodopsin, in its dark state, folds in a conformation that appears to be comparable to that of meta Ⅱ, given that it can be recognized by rhodopsin kinase even in the absence of light.

Arrestin-1 was purified to homogeneity by using three consecutive chromatography steps: (1) a DEAE-cellulose column; (2) a heparin-sepharose column that was eluted with a gradient of phytic acid; and (3) a second heparin-sepharose column that was eluted with 400 mmol/L NaCl[21]. Figure 4C shows the protein profile obtained after the last chromatography step. The elution of arrestin-1 was evaluated by SDS-PAGE analysis, which showed a polypeptide band with an apparent molecular mass of approximately 50 kDa (Figure 4C, Inset, top). This band was specifically recognized by anti-arrestin-1 polyclonal antibodies (Figure 4C, Inset, bottom). The ability of arrestin-1 to interact with the reconstituted rhodopsin and rhodopsin analogues was then evaluated by an affinity binding procedure. Arrestin-1 was incubated with rhodopsin, isorhodopsin or the 13-*cis*-retinal-rhodopsin, which were reconstituted using washed ROS membranes. Experiments were performed both in the dark and under illumination, and using phosphorylated and non-phosphorylated pigments. An experiment using opsin was also included as a control. After centrifugation, the resulting supernatants and pellets of all the samples were separated by SDS-PAGE. The interaction between arrestin-1 and the three pigments was determined qualitatively by

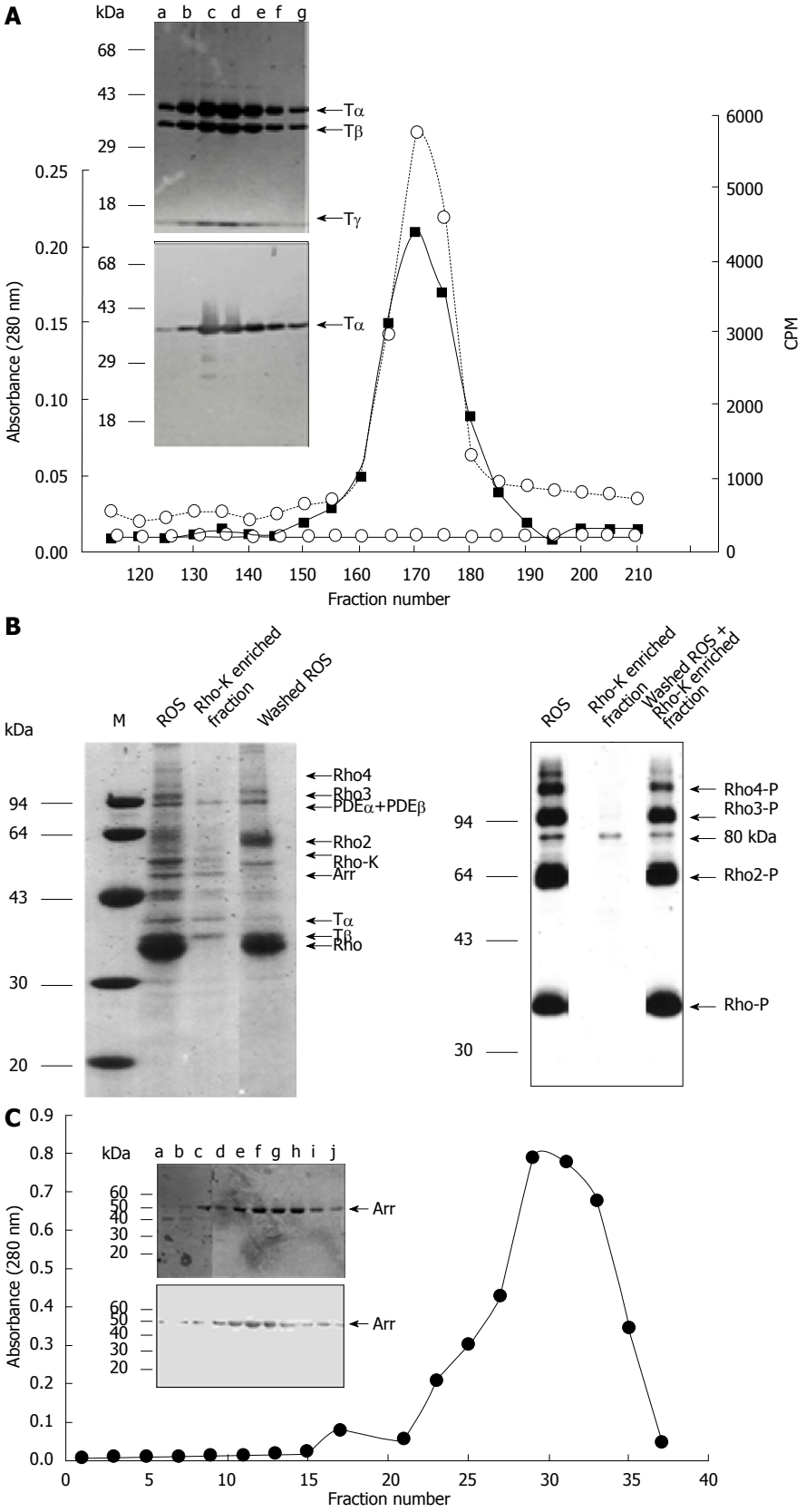


**Figure 4 Purification of transducin and arrestin-1, and preparation of an enriched fraction of rhodopsin kinase.** A: Transducin was purified to homogeneity on a DEAE-cellulose column. The elution profile was monitored at 280 nm (■). Fractions were analyzed for [8-$^{3}$H] GMPpNp binding activity (CPM) in the absence (○, continuous line) or presence (○, dashed line) of light-activated rhodopsin (as dark-depleted ROS membranes). Fractions were also examined by SDS-PAGE (Inset, top) and Western blot using anti-transducin polyclonal antibodies (Inset, bottom). Lanes a, b, c, d, e, f, and g correspond to column fractions Nº 155, 160, 165, 170, 175, 180 and 185, respectively. Arrows indicate the migration of α-, β- and γ-subunits of transducin (Tα, Tβ and Tγ); B: Autoradiography showing the light-induced *in vitro* phosphorylation of rhodopsin by rhodopsin kinase (Rho-K). Left, Coomassie blue staining; Right, Autoradiography. Samples of intact ROS membranes, a partially purified fraction of Rho-K, or a mixture of dark-depleted ROS membranes together with the enriched fraction of Rho-K were incubated with [γ-$^{32}$P] ATP under light conditions as described in Materials and Methods. Arrows indicate the migration of phosphorylated rhodopsin (Rho), rhodopsin dimers (Rho2), rhodopsin trimers (Rho3) and rhodopsin tetramers (Rho4). A polypeptide band of 80 kDa was phosphorylated in the Rho-K enriched fraction. M: Molecular weight markers; C: Arrestin-1 was purified to homogeneity after three consecutive chromatography steps, a DEAE-cellulose column, a heparin-sepharose column eluted with a gradient of phytic acid, and a second heparin-sepharose column eluted by increasing the salt concentration in the buffer. Shown is the elution profile of the last heparin-sepharose column, which was monitored at 280 nm (•). Fractions were inspected by SDS-PAGE (Inset, top) and Western blot using anti-arrestin-1 polyclonal antibodies (Inset, bottom). Lanes a, b, c, d, e, f, g, h, i, and j correspond to column fractions Nº 17, 21, 23, 25, 27, 29, 31, 33, 35, and 36. Arrows indicate the migration of arrestin-1 (Arr). ROS: Rod outer segments.

measuring the amount of arrestin-1 that was translocated from the initial mixture to the pellet. No arrestin-1 was bound to non-phosphorylated apoprotein opsin in the dark or light (Figure 7A and B, lane P). Moreover, as seen in the same figure (Figure 7A and B, lane P), no arrestin-1 interacted with phosphorylated opsin in the dark or light. Basal amounts of arrestin-1 interacted with rhodopsin, isorhodopsin and the 13-*cis*-retinal-rhodopsin complex, both in the dark and under illumination (Figure 7A and B, lane P), and as expected, all reformed photolyzed and

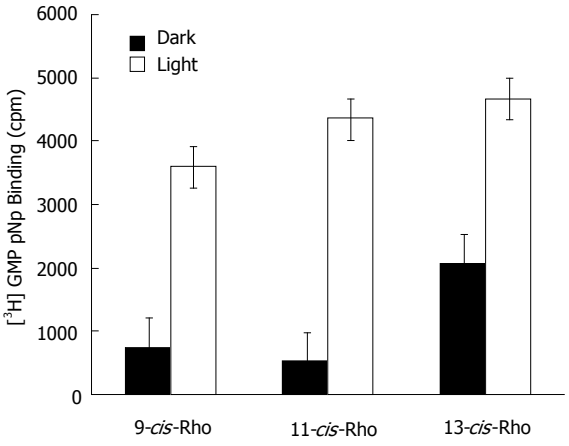


**Figure 5 Activation of transducin by reconstituted rhodopsin and rhodopsin analogues.** Binding of guanine nucleotides to transducin was evaluated by Millipore filtration using [8-$^3$H] GMPpNp. Dark and white bars correspond to experiments performed under dark and light conditions, respectively. Duplicate assays of three independent experiments were carried out. Mean ± SD are reported. Differences with *P*-values < 0.05 were considered significant.

phosphorylated pigments were capable of recognizing and binding arrestin-1 (Figure 7B, lane P). Although only residual arrestin-1 was bound to phosphorylated rhodopsin and phosphorylated isorhodopsin in the dark (Figure 7A, lane P), arrestin-1 was efficiently recognized by phosphorylated 13-*cis*-retinal-rhodopsin in the dark (Figure 7A, lane P). The silver stained gels shown in Figure 7A and B were quantified by densitometry (Figure 7C) and confirmed the results described above. The amount of reconstituted pigment, instead of the total amount of protein, was used to normalize the reported values shown in the histograms. The interaction of arrestin-1 with phosphorylated 13-*cis*-retinal-rhodopsin in the dark is consistent with our findings using transducin and rhodopsin kinase, that suggest that the rhodopsin analogue containing the 13-*cis* isomer of retinal exists as a pseudo-active state even without illumination.

## DISCUSSION

To study ligand binding pockets in proteins, specific analogues with systematically altered chemical property in their structural moieties have usually been employed to establish structure-activity relationships with regard to their functional groups. Retinal has four C = C double bonds that give rise to the four mono-*cis* isomers, the 7-*cis*, 9-*cis*, 11-*cis* and 13-*cis* forms. These isomers undergo *cis*–*trans* isomerization upon photoexcitation. The chromophore of rhodopsin is 11-*cis*-retinal and, thus, in its absence, opsin is not photosensitive and no visual function exists. Here, the 9-*cis* and 13-*cis* retinal isomers have been used to probe the rhodopsin chromophore binding pocket and to study ligand-protein relationships to better understand the photochemical *cis-trans* isomerization of rhodopsin.

The production of 11-*cis*-retinal occurs in the retinal pigment epithelium. One of the more abundant proteins in this tissue is RPE65, which has been shown to be essential for the conversion of all-*trans*-retinyl ester to 11-*cis*-retinol[32]. Leber's congenital amaurosis, a childhood blinding disorder, results from disruption of a number of genes, but in many cases, the gene for RPE65 is defective[33-36]. When RPE65 is mutated or lacking, as in the RPE65 knockout mouse and Leber's congenital amaurosis, visual function is impaired[32]. However, in the RPE65 knockout mouse, where synthesis of 11-*cis*-retinal does not occur, a minimal visual response from rod photoreceptors is obtained, which is mediated by isorhodopsin, the rod pigment formed with 9-*cis*-retinal, rather

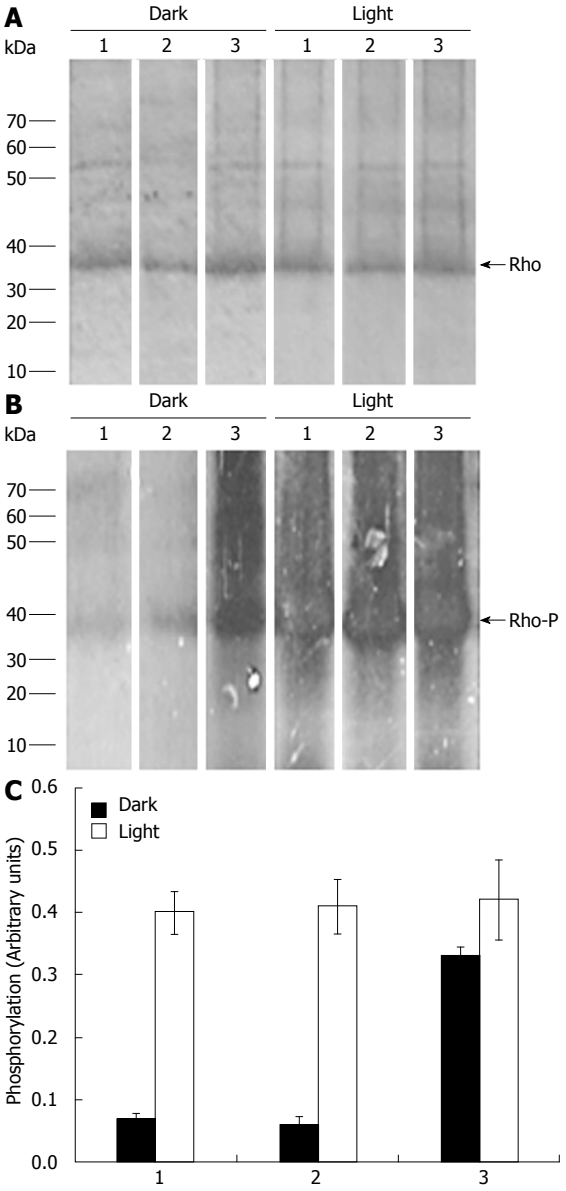


**Figure 6 Ability of the reconstituted rhodopsin and rhodopsin analogues to serve as substrates for rhodopsin kinase.** A: Coomassie blue staining; B: Autoradiography. Arrows indicate the migration of rhodopsin (Rho) and phosphorylated Rho (Rho-P); C: Densitometry of the autoradiograms shown in B. Dark and white bars correspond to experiments performed under dark and light conditions, respectively. Mean ± SD of three independent experiments are reported. Differences with P-values below 0.05 were considered significant.1: 9-*cis*-Rho; 2: 11-*cis*-Rho; 3: 13-*cis*-Rho.

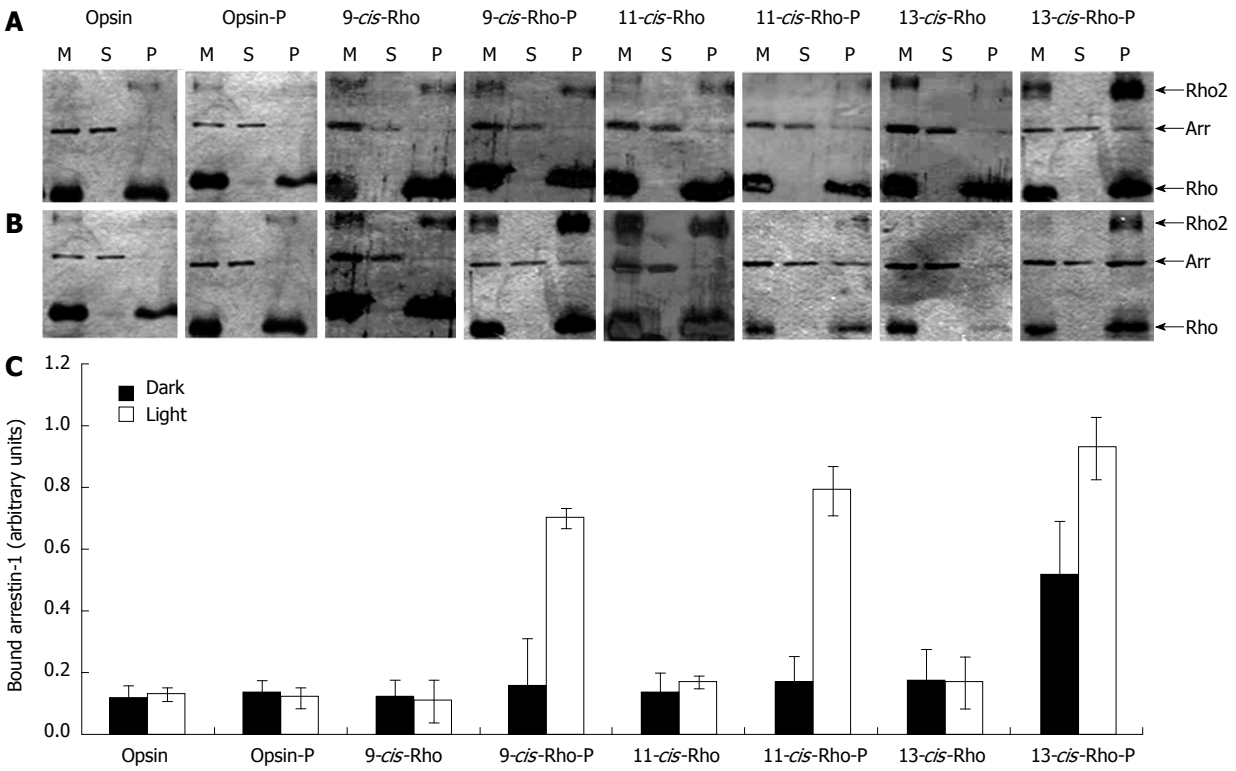


**Figure 7 Ability of arrestin-1 to interact with the reconstituted rhodopsin and rhodopsin analogues.** Arrestin-1 was combined with membranes containing opsin, phosphorylated opsin (opsin-P), isorhodopsin (9-*cis*-Rho), phosphorylated isorhodopsin (9-*cis*-Rho-P), rhodopsin (11-*cis*-Rho), phosphorylated rhodopsin (11-*cis*-Rho-P), 13-*cis*-retinal-rhodopsin (13-*cis*-Rho), and phosphorylated 13-*cis*-retinal-rhodopsin (13-*cis*-Rho-P), under dark (Panel A) and light (Panel B) conditions. The mixtures (M) were centrifuged and aliquots of each mixture and of the resulting supernatants (S) and pellets (P) were separated by SDS-PAGE. Gels were colored by silver staining. In Panel C, the amount of arrestin-1 that interacted with the phosphorylated pigments in the pellet fraction was quantified by densitometry. Mean ± SD of three independent experiments are reported. Differences with *P*-values < 0.05 were considered significant Arrows indicate the migration of rhodopsin (Rho), arrestin-1 (Arr), and rhodopsin dimers (Rho2).

than rhodopsin[13]. Isorhodopsin, is photosensitive and appears to be very similar to rhodopsin, as determined in numerous *in vitro* studies and experiments using intact retinae and isolated photoreceptors[37,38]. Then, although endogenous 9-*cis*-retinal has not been reported in the retina, the high expression of 9-*cis*-retinol dehydrogenase (RDH4/RDH5) in the retinal pigment epithelium[39,40] suggests that 9-*cis*-retinal could be generated in that tissue. Actually, 9-cis-retinoids do exist in many tissues, with highest concentrations in liver and kidney, and are essential for gene regulation, growth and development[41,42]. In contrast, the 13-*cis* configuration of retinal has never been observed in vision and as such is not physiologically relevant in the visual process. Nevertheless, all-*trans*-retinal is an essential component of type I , or microbial, opsins such as bacteriorhodopsins, channelrhodopsins, sensory rhodopsins and halorhodopsin. Type I opsin genes are found in prokaryotes, algae, and fungi, where they control diverse functions such as phototaxis, energy storage, development, and retinal biosynthesis[43]. Using microbial opsin genes, prokaryotes can transduce light to shift proton gradients, modulate chloride balance, or switch flagellar motor direction, whereas motile algae transduce light to change flagellar beating to direct locomotion toward environments optimally illuminated for their photosynthetic requirements. In these seven-trans-membrane-segment receptor proteins, light causes the all-*trans*-retinal to become 13-*cis*-retinal, which then cycles back to all-*trans*-retinal in the dark state. Unlike the situation with rhodopsin, in which the retinal-protein linkage is hydrolyzed after photoisomerization[44], the activated retinal molecule in type I opsins, 13-*cis*-retinal, does not dissociate from its opsin protein, but thermally reverts to the all-*trans* state while maintaining a covalent bond to its protein partner[45]. Accordingly, 13-*cis*-retinal is physiologically crucial in those organisms that possess type I opsins.

The regular instability of 11-*cis*-retinal limits its commercial availability. The standard procedure used to prepare 11-*cis*-retinal consist of an isomerization reaction of all-*trans*-retinal by irradiation under 436 nm[46-48], which generates a mixture of 9-*cis*-retinal, 11-*cis*-retinal and 13-*cis*-retinal that requires to be separated by chromatography techniques, such as alumina column chromatography, thin-layer chromatography, high-performance liquid chromatography (HPLC), or flash countercurrent chromatography (FCCC). Photochemical and enzymatic processing of retinoids in the eye is essential for perception of the light signal and for sustaining vision by regeneration of visual pigments[12]. Specifically, the photoisomerized all-*trans*-retinal is converted back to the 11-*cis*-retinal chromophore by an enzymatic pathway of chemical

reactions termed the retinoid cycle[11,12]. Why the 9-*cis*- and 13-*cis*-isomers of retinal are not formed in the eye in addition to 11-*cis*-retinal? The retinal G protein-coupled receptor (RGR) is a protein that structurally resembles visual pigments and other G protein-coupled receptors. RGR appears to play a role as a photoisomerase in the production of 11-*cis*-retinal. The proposed function of RGR, in a complex with 11-*cis*-retinol dehydrogenase (RDH5), is to regenerate 11-*cis*-retinal under light conditions[49]. Maeda *et al*[50] evaluated the role of RGR using RGR single knockout mice, and RGR and RDH5 double knockout mice, under various conditions. The most striking phenotype of RGR knockout mice after illumination included light-dependent formation of 9-*cis*- and 13-*cis*-retinoid isomers. These isomers were not formed in wild-type mice because either all-*trans*-retinal is bound to RGR and protected from isomerization to 9-*cis*- or 13-*cis*-retinal or because RGR is able to eliminate these isomers directly or indirectly. These results suggest that RGR and RDH5 are likely to function in the retinoid cycle.

In the present manuscript, we focused on comparing the interactions of rhodopsin and rhodopsin analogues containing 9-*cis*-retinal and 13-*cis*-retinal with other proteins of the visual cascade, such as transducin, rhodopsin kinase and arrestin-1. Under dark conditions, 13-*cis*-retinal-rhodopsin was capable of catalyzing transducin GDP/GTP exchange and was highly phosphorylated by rhodopsin kinase. Since 13-*cis*-retinal-rhodopsin behaves like active rhodopsin independently of light, and given that both transducin activation and phosphorylation by rhodopsin kinase require the generation by photolysis of the meta II intermediate of rhodopsin, we propose that the structure of dark 13-*cis*-retinal-rhodopsin adopts a tridimensional conformation that mimics the active photoproduct of rhodopsin. Moreover, arrestin-1 was also efficiently recognized by phosphorylated 13-*cis*-retinal-rhodopsin in the dark. As shown by Gurevich *et al*[24], arrestin-1 binds phosphorylated light-activated rhodopsin with remarkable selectivity. However, arrestin-1 binding to an equal amount of dark (inactive) phosphorylated rhodopsin or active unphosphorylated rhodopsin (light-activated rhodopsin) is 10-20 times lower, whereas its binding to inactive unphosphorylated rhodopsin is barely detectable[24]. Thus, rhodopsin activation or phosphorylation alone promotes relatively weak arrestin-1 interaction. In addition, arrestin-1 binding to phosphorylated light-activated rhodopsin is many times greater than the sum of dark phosphorylated rhodopsin and light-activated rhodopsin levels, suggesting that the binding mechanism is more sophisticated than a simple cooperative two-site interaction. Gurevich *et al*[24] proposed a model positing that arrestin-1 has two sensor sites, an activation sensor that binds receptor elements that change conformation upon activation, and a phosphate sensor that binds receptor attached phosphates. When the receptor is phosphorylated and active at the same time, both sensors bind. Simultaneous engagement of the two sensor sites allows arrestin-1 transition into the active high affinity receptor-binding state. Since the conformation of dark 13-*cis*-retinal-rhodopsin appears to mimic the structure of the meta II photointermediate, phosphorylated 13-*cis*-retinal-rhodopsin seems to be sufficient to be recognized by arrestin-1 even in the absence of light.

Since the 9-*cis*, 11-*cis*, and 13-*cis* isomers of retinal are not planar, changes at the *cis* configuration in the polyene structure may cause important non-planar distortions in the retinal molecule that in turn may affect its longitudinal size. Employing the molecular orbital program MOPAC (version 1.11), we determined the structures of minimal energy for the various retinal isomers used here. The distances from carbon C-2 to carbon C-15 were found to be 10.84 Å, 10.96 Å, and 11.54 Å for 11-*cis*-retinal, 9-*cis*-retinal, and 13-*cis*-retinal, respectively. The retinal molecule reaches its longest longitude in its all-*trans* configuration (13.02 Å). A clear relationship between the size of each isomer and its accessibility to the chromophore binding pocket in the apoprotein opsin can be established when these theoretical distances were taken in consideration and contrasted with the percentage of pigment that was regenerated with each retinal isomer. 11-*cis*-Retinal and 13-*cis*-retinal, which corresponded to the shortest and longest isomers, showed the highest and lowest percentage of pigment reconstitution, respectively. Thus, it is evident that some size restrictions exist within the prosthetic group binding site. In addition, structural differences may occur when the various retinal isomers are incorporated and accommodated into the apoprotein to reform the distinct pigments.

It is known that the spectral properties of 9-*cis*-retinal, 11-*cis*-retinal, and 13-*cis*-retinal are very similar; all three compounds show absorption maxima at 365-370 nm. Interaction between 11-*cis*-retinal and opsin generates the ground state of rhodopsin with its characteristic peak at about 500 nm. The red shift of 11-*cis*-retinal in rhodopsin is a result of the protonated Schiff base linkage between the aldehyde and the ε-amino group of Lys296, which is stabilized by the Glu113 counter-ion. Moreover, the positive charge is delocalized through the polyene moiety of retinal. Rhodopsin is constrained in an inactive conformation because binding of 11-*cis*-retinal to Lys296 *via* the protonated Schiff base induces changes in rhodopsin' s helical transmembrane domain and cytoplasmic surface that prevent interaction with native transducin, rhodopsin kinase and arrestin-1. Upon photoisomerization of 11-*cis*-retinal to all-*trans*-retinal, the receptor undergoes major structural rearrangements that include displacement of the positively charged Schiff base from its interaction with negatively charged Glu113. Based on this mechanism of action, a bulky ligand might affect and modify the regular distance between the Glu113 counter-ion and the retinal attachment site in the protein, affecting in turn the spectroscopic properties of the regenerated pigment. Blue shifts in isorhodopsin ($\lambda_{max}$ = 486 nm) and 13-*cis*-retinal-rhodopsin ($\lambda_{max}$ - 467 nm) correlate well with the increase in longitudinal size of 9-*cis*-retinal and 13-*cis*-retinal compared to 11-*cis*-retinal. During the rho-

dopsin photocycle, the protein relaxes through a series of distinct photointermediates, each with characteristic UV/visible absorption maxima. Most of these intermediates can only be trapped by using ultra freezing temperatures. The metarhodopsin I photointermediate (meta I), which is the inactive precursor of meta II, possesses a characteristic peak at 478 nm. Interestingly, the spectroscopic properties of 13-*cis*-retinal-rhodopsin in the dark were comparable to those of meta I. The resemblance of meta I and the 13-*cis*-retinal-rhodopsin pigment might cause the pseudo-activation state seen for the latter even without illumination. Since meta I can only be generated following freeze-trapping at -40 ℃, and at temperatures below the phase transition temperature of the surrounding lipids, it was not viable for us to carry out a direct comparison between the properties of meta I and the 13-*cis*-retinal-rhodopsin analogue.

Rhodopsin pigment regeneration studies using available retinal isomers showed that stable isomeric pigments can be formed using a diversity of isomers such as 11-*cis*, 9-*cis*, 7-*cis*, 9,13-*dicis*, 7,13-*dicis*, 9,11-*dicis*, 7,11-*dicis*, 7,9-*dicis*, 7,9,11-*tricis*, 7,9,13-*tricis*, *etc*., with varying rates of pigment formation[51]. With the exception of 9-*cis*-retinal, all isomers required much longer times to give isomeric pigments at reduced yields[51]. By using the crystal structures of rhodopsin, Liu *et al*[52] reproduced the binding cavity of rhodopsin containing the 11-*cis*-retinal, and examined whether other isomers were capable of being accommodated within the pocket. When the 9-*cis* and 7-*cis* isomers of retinal were tested it was clear that all atoms of the two isomeric pigment analogs fitted well within the binding cavity. However, when the pigment was replaced with atoms of the 13-*cis* protonated Schiff base, it was clear that the 13-methyl group and partly C13 and C14 of the 13-*cis* chromophore was projected far beyond the binding pocket overlapping with atoms in the β-sheet of the loop that connects the TM4 and TM5 helices[52]. These results confirm that steric restrictions exist in the binding cavity and explain previous reports showing nonbinding of the 13-*cis* or all-*trans* isomers to the inactive state of the protein[16], as well as our results that showed a low percentage of regeneration of the 13-*cis*-retinal-rhodopsin analog. The much reduced rate for pigment formation for the 13-*cis* isomer and other retinal isomers is likely due to the altered ring conformations, the relocated 9-methyl groups, and shifts of the polyene chain.

The C-9 and C-13 methyl groups of the 11-*cis*-retinal appear to be pivotal elements in ligand-receptor communication. For instance, 9- and 13-demethylretinals yielded analogue pigments, but with an increase in constitutive activity and/or much reduced physiological activity[53,54]. Ebrey *et al*[55] observed that 13-demethyl-rhodopsin, which is opsin regenerated with 11-*cis*-13-demethyl-retinal, activated transducin as measured by cGMP-phosphodiesterase PDE6 activity in the dark. This finding was surprising, since 13-demethyl-retinal lacks only the methyl group in position 13. However, the 9-*cis* isomer of 13-demethylretinal like all the other activating pigments required light[55]. When, 11-*cis*-13-demethyl-retinal was preincubated with opsin in the dark, significant phosphorylation was observed[56]. The activity was increased when the all-*trans* isomer was used, but decreased with 9-*cis*-13-demethyl-retinal. The results obtained by Buczyłko *et al*[56] were consistent with the observations of Ebrey *et al*[55]. Deletion of methyl groups to form 9-demethyl and 13-demethyl analogues, as well as addition of a methyl group at C10 or C12, shifted the meta I/meta II equilibrium toward meta I, such that the retinal analogues behaved like partial agonists[54]. To examine the steric limits of the 9-methyl and 13-methyl binding pocket of opsin, deGrip *et al*[57] prepared cyclopropyl and isopropyl derivatives of 11-*cis*- and 9-*cis*-retinal, at C-9 and C-13, and of α-retinal at C-9. Most isopropyl analogues showed very poor binding, whereas most cyclopropyl derivatives exhibit intermediate binding activity. The data of deGrip *et al*[57] were in line with the growing body of evidence showing that the interplay between a receptor and its ligand is very finely tuned. Small modification of a ligand can already alter this interplay and thereby redirect the conformational space of a receptor, leading to a different activity profile. Here we have shown that 13-*cis*-retinal-rhodopsin behaves as a pseudo-active pigment in the dark. Similar to 11-*cis*-13-demethyl-retinal-rhodopsin, the structure of 13-*cis*-retinal-rhodopsin probably embraces a tridimensional conformational fold that mimics to some extent the active meta II photointermediate of rhodopsin. Consequently, 13-*cis*-retinal-rhodopsin is capable of interacting with transducin, rhodopsin kinase and arrestin-1 even without illumination. Palczewski *et al*[58] have also shown that active pseudo-photoproducts, which stimulate transducin activation and opsin phosphorylation by rhodopsin kinase, are formed with opsin and retinal analogues lacking the 13 methyl or the terminal two carbons of the polyene chain as well as with opsin and all-*trans*-retinal. Other reports have also shown that an activated receptor may be generated without illumination by addition of all-*trans*-retinal or its analogues to opsin[56,59-61]. Cohen *et al*[59] found that transducin activation by the all-*trans*-retinal-opsin complex was strongly pH-dependent with the most efficient catalysis at pH = 5-6. Hofmann *et al*[60] demonstrated that free all-*trans*-retinal can react with the apoprotein to form pseudo-photoproducts that are spectrally identical to the photoinduced metarhodopsin species (meta I/II/III). By measuring the increased phosphorylation of opsin by rhodopsin kinase, Buczyłko *et al*[56] showed that the potency of stimulation depended on the chemical and isomeric nature of the analogues and the length of the polyene chain. For example all-*trans*-C17 aldehyde was the most effective in stimulation of opsin phosphorylation, while longer (all-*trans*-retinal) and shorter analogues (all-*trans*-C15 aldehyde) were less potent. All-*trans*-C22 aldehyde was not effective suggesting that the length of this retinoid excluded it from the binding to opsin, while the shortest aldehyde, all-*trans*-C12 aldehyde, was only modestly effective. This specificity suggested a unique inter-

action of opsin with retinoids, rather than a nonspecific lipid-like effect or interaction with peripheral amines[56]. Ligand-free opsin is also capable of activating transducin, although at a much reduced level than light-activated rhodopsin[61,62], but this activity was enhanced by a factor of about 10 by the presence of all-*trans*-retinal. Interestingly, when the sizes of the various isomers of retinal used in the present work were compared, 13-*cis*-retinal was more alike to all-*trans*-retinal than 9-*cis*-retinal or 11-*cis*-retinal.

Various tridimensional conformations of the photoreceptor protein have been solved. Park *et al*[63] reported the X-ray crystal structure of ligand-free native opsin from bovine retinal rod cells. Compared to rhodopsin[64], opsin shows prominent structural changes in the conserved E(D)RY and NPxxY(x)$_{5,6}$F motifs and in the transmembrane fifth to transmembrane seventh regions (TM5-TM7). These structural changes reorganize the empty retinal-binding pocket to disclose two openings that may serve for the entry and exit of retinal. The lack of the interacting prosthetic group causes distinct structural alterations in the retinal-binding pocket. For example, part of the space occupied by the β-ionone ring of retinal is filled in opsin with the side chains of some aromatic residues[63]. In rhodopsin, retinal is held along the polyene chain by amino acids located in TM3, TM6 and loop E2[64]. In opsin, the extracellular part of TM3 and loop E2 are slightly moved away from helices TM5-TM7. Thereby, the retinal-binding pocket becomes wider towards the retinal attachment site in Lys296, and the ε-amino group of Lys296 does not seem to be involved in a salt bridge with Glu113, which corresponds to the retinal Schiff base counter-ion in the rhodopsin dark state, or with Glu181, which was proposed to be part of a complex counter-ion which forms in meta I [65]. Moreover, it has been shown that opsin can readily adopt inactive and active conformations *in vitro*, and low pH and a synthetic peptide derived from the C terminus of the α-subunit of transducin stabilized this active conformation of opsin[66]. Scheerer *et al*[67] reported the crystal structure of the complex between active opsin and the carboxy terminus peptide of the α-subunit of transducin, and clear conformational differences can be detected when the structures of inactive and active opsin are compared. More recently, Choe *et al*[68] used the low pH induced-active conformation of opsin to obtain crystals of meta Ⅱ, by soaking crystals of active opsin with all-*trans*-retinal. They presented the crystal structures of meta Ⅱ alone or in complex with a C-terminal fragment derived from the α-subunit of transducin. The binding site for all-*trans*-retinal appears to be preformed in the active conformation of opsin because the presence of retinal in the meta Ⅱ structures causes only a small adjustment of some amino acid side chains[68], while the Lys296 side chain, which is more flexible in ligand-free opsin[63], becomes ordered due to its linkage with retinal. From the crystal structures of rhodopsin, opsin, activated opsin and meta Ⅱ, it is clear that changes in the prosthetic group binding pocket occur in each of the different conformations of the protein, and receptor can make use of the conformational flexibility of the ligand and the variability of its interaction with the binding site.

## ACKNOWLEDGMENTS

We want to thank Dr. Debra Thompson, University of Michigan, United States for supplying the 11-*cis*-retinal used in this work.

## COMMENTS

### Background

G protein-coupled receptors activate signaling paths in response to a diverse number of stimuli such as photons, $Ca^{2+}$, organic odorants, amines, hormones, nucleotides, nucleosides, peptides, lipids and even large proteins. The dim-light photoreceptor rhodopsin is a highly specialized G protein-coupled receptor composed of the apoprotein opsin, and a covalently linked 11-*cis*-retinal chromophore.

### Research frontiers

Little is known about the interactions of rhodopsin analogues with other proteins in the visual cascade. Although, it has been reported that the retinal binding site in the inactive state of rhodopsin can accommodate the 7-*cis*, 9-*cis* and 11-*cis* isomers of retinal, but not the longer all-*trans* or 13-*cis* isomers[16], in the present work we were able to reconstitute a rhodopsin analogue containing 13-*cis*-retinal, in addition to photoreceptor proteins containing 9-*cis*-retinal and 11-*cis*-retinal.

### Innovations and breakthroughs

This study compared the binding of reconstituted rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin to transducin, rhodopsin kinase and arrestin-1, both in the dark and under illumination.

### Applications

The rhodopsin analogue containing the 13-*cis* isomer of retinal was capable of activating transducin and was highly phosphorylated by rhodopsin kinase independently of light. Arrestin-1 was also efficiently recognized by phosphorylated 13-*cis*-retinal-rhodopsin in the dark.

### Peer review

This manuscript by Araujo *et al* is aimed to study if reconstituted rhodopsin, 9-cis-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin interact with transducin, rhodopsin kinase and arrestin-1. The authors isolated rod outer segments (ROS) from bovine retinas, generated rhodopsin and rhodopsin analogues with the different retinal isomers, purified transducin and arrestin-1 to homogeneity, and obtained an enriched-fraction of rhodopsin kinase by extracting freshly prepared ROS. The authors characterized the reconstituted rhodopsin and rhodopsin analogues through three sets of experiments: activation of transducin, ability to serve as substrates for rhodopsin kinase, and binding to arrestin-1. Different approaches including column chromatography, guanine nucleotide binding assay, *in vitro* phosphorylation, *etc.* were used. They found that rhodopsin analogue harboring the 13-*cis* isomer of retinal is capable of activating transducin in a light-independent way. They concluded that the rhodopsin analogue containing the 13-*cis* isomer of retinal seems to fold in a pseudo-active conformation that mimics the active photointermediate of rhodopsin.

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