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In Vitro Assays of Rod and Cone Opsin Activity: Retinoid Analogs as Agonists and Inverse Agonists

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Abstract

Upon absorption of a photon, the bound 11-*cis*-retinoid isomerizes to the all-*trans* form resulting in a protein conformational change that enables it to activate its G protein, transducin, to begin the visual signal transduction cascade. The native ligand, 11-*cis*-retinal, acts as an inverse agonist to both the apoproteins of rod and cone visual pigments (opsins); all-*trans*-retinal is an agonist. Truncated analogs of retinal have been used to characterize structure–function relationships with rod opsins, but little has been done with cone opsins. Activation of transducin by an opsin is one method to characterize the conformational state of the opsin. This chapter describes an in vitro transducin activation assay that can be used with cone opsins to determine the degree to which different ligands can act as an agonist or an inverse agonist to gain insight into the ligand-binding pocket of cone opsins and differences between the different classes of opsins. The understanding of the effects of ligands on cone opsin activity can potentially be applied to future therapeutic agents targeting opsins.

Keywords

Retinal analog; cone opsin; G protein-coupled receptor; transducin; cone pigment; rhodopsin

1. Introduction

There are two types of photoreceptors in vertebrate retina – rods and cones. They have distinct physiological roles, the rods operating under dim light conditions and being exquisitely sensitive and the cones requiring more light and discerning colors. The cones are required for normal human vision. The light-detecting components in the photoreceptor cells are visual pigments. Visual pigments are comprised of proteins (opsins) and chromophore (11-*cis*-isomer of vitamin A aldehyde (retinal) or 3,4-dehydroretinal). Because there is no variation in the chromophore, the ability to detect light across the color spectrum depends on the influence of the different opsins on the absorption properties of the chromophore. Retinal analogs have been useful in the past to probe spectral tuning and the binding site restraints of visual pigments (1).

The protein moieties of rod and cone pigments (opsins) are highly homologous to each other and also belong to the superfamily of G protein-coupled receptors (GPCRs). The G protein activated by these visual pigments in initiating the visual signal transduction cascade is transducin. An opsin is referred to as being active when it is able to activate this G protein.

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¹⁰If the analog to be tested results in generation of a stable pigment, then the pigments can be detergent solubilized and immunopurified allowing for spectroscopic and activity measurements on the same samples. Such was done with 9-demethylretinal and salamander rod and cone opsins (16). In these situations, the detergent choice and concentration can greatly affect transducin activation assays. CHAPS is not a good detergent for transducin activation assays. Dodecylmaltoside, if the final concentration is kept at or below 0.1%, is a suitable detergent (29).

Unlike other GPCRs, the ligand of visual pigments is covalently bound to a strictly conserved lysine in the seventh transmembrane helix of the opsins through a Schiff base linkage. 11-*cis*-Retinal (or the 3,4-dehydro form) acts as an inverse agonist with all the vertebrate visual opsins tested, maintaining the receptor in an inactive state. On absorption of a photon, bound 11-*cis*-retinal isomerizes to the more stable all-*trans* form and the protein receptor is transformed into an active conformation. Thus, it is the light that converts the inverse agonist into an agonist via photoisomerization. In the eye, the Schiff base between the all-*trans*-retinal and the protein is subsequently hydrolyzed and the retinal is reduced to all-*trans*-retinol leaving the opsin as the apoprotein. The opsins themselves are weakly constitutively active (2–6) and all regenerate in the presence of 11-*cis*-retinal, reforming the inactive, photosensitive pigments.

Although the native ligand is covalently bound to the opsins in the inactive and photoactivated states, a covalently attached ligand is not absolutely required to deactivate or activate the opsins. Several truncated analogs of retinal have been demonstrated to activate the rod opsin (7–9). Furthermore, a highly constitutively active rhodopsin mutant where the conserved lysine that normally forms the Schiff base with the chromophore has been mutated has been shown to be deactivated and made light-sensitive with the 11-*cis*-retinyl Schiff base, where 11-*cis*-retinal has been coupled to *n*-propylamine (10).

To date, there has been a dearth of information of ligand-dependent activation and deactivation of cone opsins. A major reason for this is the lack of methods and sources for pure cone opsins; whereas, rod opsins in good purity are easily isolated. Another reason is the perceived instability of the protein (11). Cone pigments have been shown to lose its chromophore or, in the presence of analogs, exchange chromophores in the dark (12–14) unlike the rod opsin where the pigment (rhodopsin) is quite stable even to hydroxylamine.

Heterologously expressed opsins are a convenient tool for probing opsin–ligand interactions as there is no question of retinoid photoproducts remaining attached to the membranes, there is no mixture of different opsins, and mutants can be readily constructed and tested. We have shown that different ligands can affect the cone opsins and pigment activation differently depending on opsin type and ligand (4, 15, 16). All-*trans*-retinal and all-*trans*-retinol are both agonists with all four opsin groups (unpublished results, 2009). The other physiologically relevant ligand is 11-*cis*-retinol. Surprisingly, this ligand has quite different activities with the various opsins which have important physiological consequences (15, 17, 18). β -Ionone, representing a fragment of retinal, has been shown to be both an inverse agonist and agonist depending on cone opsin type (4), illustrating that cone opsins do not all interact with ligands in the same manner.

In Fig. 4.1, we illustrate the modulation of transducin activation by expressed human red cone opsin as a function of retinal analog length. This methodology provides a convenient in vitro tool for studying the interactions of opsins with various compounds that are potential ligands for these opsins. For the human red cone opsin, as the polyene chain decreases in length, the ligand converts from an agonist activating the G protein to an inverse agonist, decreasing the opsin's ability to activate this G protein (Fig. 4.1b).

We describe here an in vitro assay for determining the ability of a retinal analog to act as an agonist or an inverse agonist with various opsins. The use of this assay can serve to provide insight into structural and functional similarities and differences among cone opsins.

2. Materials

2.1. Material Sources

GTP γ S-35: catalog number NEG030H250UC, PerkinElmer Life and Analytical Sciences, Waltham, MA; 1D4 antibody – available through a number of vendors including catalog number MA1-722 from Affinity BioReagents/Thermo Fisher Scientific, Rockford, IL.

2.2. Stock Solutions

1. Membrane prep buffer: 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.1 mM EDTA, 10 mM Tris–HCl (pH 7.4). The opsin concentration is typically 10–50 nM (*see* Note 1).
2. Transducin buffer (2×): 20 mM Tris (pH 7.4), 4 mM MgCl₂, and 2 mM DTT.
3. Assay buffer (10×): 100 mM MES buffer, 1 M NaCl, 50 mM MgCl₂ at pH 6.5.
4. DTT solution: 50 mM in Milli-Q water.
5. Analog solution: 20 mM in ethanol (*see* Note 2).
6. GTP γ S solution: 150 μ M cold GTP γ S with GTP γ S³⁵ at ~0.25 mCi/ml; 100 μ l of a 150 μ M solution of GTP γ S from ~3 mM stock solution and add 2 μ l (25 μ Ci) GTP γ S-35.
7. Assay rinse buffer: 10 mM Tris, pH 6.4, 100 mM NaCl, 5 mM MgCl₂.

3. Methods

3.1. Membrane Preparation Containing Opsins

1. Transiently expressed cone opsins in COS cells (19) with opsin genes with the codons for at least the last eight amino acid residues of bovine rhodopsin, the 1D4 epitope (*see* Note 3).
2. COS cell membranes containing the opsins isolated using a discontinuous sucrose gradient (5, 20, 21).
3. Membrane suspensions of 25 μ M aliquots in membrane prep buffer stored at –80°C.
4. The amount of opsin in the membrane preparations are determined by slot blot analysis (20) using known amounts of bovine rhodopsin as reference and probed with the rhodopsin 1D4 antibody.

3.2. Transducin Preparation

1. Transducin purified from bovine retinae (W.L. Lawson, Lincoln, NE) (22–24) (*see* Note 4).

¹Opsin concentrations should be kept as low as possible, typically nanomolar range, to allow for multiple turnovers.

²As a starting point to quickly assay a ligand, we have been using 200 μ M of the ligand (20 mM stock solution in ethanol, if possible). For most ligands we have tested, this is more than sufficient. However, for completeness, the ligand concentration dependence ought to be determined.

³We express opsins in COS cells. Other cells such as HEK293 (25, 26) and Sf 9 (27) cells have been used to successfully express rod and cone opsins and can certainly be used for these assays. We prefer to use COS cells because of our experience with them and relative low cost of maintenance and transfection. The DEAE-dextran transient transfection method is quite harsh but tolerated by confluent COS cells, and the reagents are relatively inexpensive and readily prepared in the lab rather than purchased from a kit. Furthermore, we passage the cells with media supplemented with bovine serum (19) rather than fetal bovine serum, which results in considerable cost savings.

2. This sample is then applied to a 3 ml DEAE-cellulose anion exchange column (22), which is washed with 10 column volumes of 1× transducin buffer and then 20 column volumes of the same buffer with 100 mM NaCl.
3. Transducin is eluted with the transducin buffer containing 500 mM NaCl and fractions monitored by absorbance at 280 nm. Pooled fractions containing transducin are dialyzed three times against a 1:1 mixture of glycerol and 2× transducin buffer, diluted to 50 μM and stored at −20°C.

3.3. Activity Assay

The ability of the specific opsin to activate bovine rod transducin is determined using a radioactive filter-binding assay with membrane preparations of opsin expressed in COS cells essentially as described previously with a few modifications (5, 21) (*see Note 5*).

1. Wet filter membranes with water in a tray; place filters onto the vacuum manifold; assemble the manifold. We use a Millipore 1225 sampling vacuum manifold (Millipore, Billerica, MA) with 25 mm diameter Millipore mixed cellulose ester membranes (HAWP 02500; Millipore, Billerica, MA) attached.
2. Prepare the reaction mixture without retinoid and GTPγS in a 1.5 ml microcentrifuge tube:

Volume (μl)	
70	Deionized water
10	10× assay buffer (<i>see Note 6</i>)
2	50 mM DTT
5	Transducin (50 μM stock)
10	Opsin/visual pigment (typically, 10–50 nM stock concentration) (<i>see Note 1</i>)

3. Add 1 μl retinal/retinal analog/ethanol (for opsin control).
4. Start reaction by adding 2 μl of 150 μM GTPγS solution and start the clock.
5. At each time point, remove 10 μl aliquots and pipet onto filter. Wash filters three times with 4 ml rinse buffer with a repeating pipettor.
6. Continue with each time point (usually 1 min intervals).
7. Transfer filter membranes into scintillation vials.
8. Add 10 μl of reaction mixture directly into scintillation vials. These counts will be used to convert counts per minute (cpm) to GTPγS amounts in pmol because these scintillation vials contain 30 pmol GTPγS since none was washed away.

⁴This is essentially a protocol for rod outer segments prepared in the light using bleached rhodopsin to anchor the transducin to the membrane and releasing transducin from the membrane with GTP.

⁵The assay is based on the finding that as the receptor (opsin) activates the G protein (transducin), a bound GDP is released and free GTPγS binds to the G protein. Proteins including transducin and transducin bound with radioactive GTPγS adhere to the filter membrane, and unbound GTPγS flows through with the wash buffer. In this manner, the rate at which GTPγS is taken up by the G protein can be determined by plotting cpm per unit time (or more appropriately picomole-bound GTP per unit time). The cpm can be converted to mol GTPγS because the amount of GTPγS in the scintillation vial(s) from step 8 is 30 pmol.

⁶The constitutive activity of the opsins is measured at an acidic pH (the final pH is 6.4 in our assays, but the 10× stock buffer is made at pH 6.5), which enhances the activation by the apoprotein such that the lower activity in the presence of inverse agonist such as 11-*cis*-retinal is clearly distinguishable (2, 4, 5).

9. Add 10 ml Amersham BCS scintillation cocktail (catalog number: NBCS104, GE Healthcare, Piscataway, NJ).
10. The vials are shaken for at least 1 h and often overnight for convenience and measured in a scintillation counter (usually 1–5 min counts). The counts per minute can be converted to pmol GTP γ S bound, which reflects the amount of transducin being activated with time (*see* Notes 5, ⁷, and ⁸).
11. Other visual pigment protein preparations can be used (*see* Notes 9–¹¹), and if a light-sensitive pigment is generated, dark/light differences can be determined (*see* Note 12).

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⁷Kinetics are generally linear as we assume pseudo-first-order kinetics. This requires the substrates to be in excess and opsin to be limiting. Deviations from linearity can occur if the photoactive intermediate is decaying rapidly compared to the timescale of the assay such as with cone pigments (16) or if other substrates are being depleted.

⁸We generally report our activities as a mean of three or more measurements $\pm$ standard error of the mean.

⁹We have described methods for purified membranes of opsins transiently expressed in COS cells. However, opsins can be purified using the 1D4 antibody coupled to Sepharose 4B to immunopurify opsins solubilized and purified with CHAPS and lipid. For example, rhodopsin mutants have been purified with CHAPS/asolectin (28). Because of the high critical micelle concentration of CHAPS, this detergent is easily removed by a number of methods to leave behind opsins in asolectin vesicles.

¹¹Native opsins can be measured. Rod outer segments are the most easily obtained from a sucrose float. However, cone pigments from cone-dominant retinas have been purified from native sources such as chicken (30) and geckos (31) and could be used. However, opsins from native sources ideally need to have the native chromophore removed to ensure that effects are due to retinal analogs and not the native chromophore.

¹²If rod and/or cone pigments are to be assayed for light-dependent activation, then light conditions must be considered. If membrane preparations are used with an excess of chromophore, the assays should be conducted in the dark (dim red light conditions) and a pulse of light used to bleach the pigment but the assay continued in the dark. Continuous light can result in photoreactions of the light-activated product and photoactivation of new pigment regenerated after hydrolysis of the chromophore. The latter is especially a concern with cone pigments as their chromophore is released in the timescale of seconds, whereas the release is several minutes with rhodopsin (16). We bleach our samples with a slide projector containing a 300-W bulb with a longpass filter attached to minimize bleaching the active intermediate. The main consideration for the filter is to overlap with the absorption spectrum of the pigment band and to minimize light hitting the near-UV spectrum. Thus the type of optical filter depends on the absorption spectrum of the pigment of interest. While there are different sources and types of optical filters available, we have purchased a number of longpass filters from Edmund Optics (Barrington, NJ) as they are quite inexpensive and available in a variety of colors and sizes. For example, the 2-in. square OG-530 longpass glass filter is convenient for rhodopsin and green and red cone pigments. Appropriate band-pass filters can also be used.

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Fig. 4.1.

Transducin activation by expressed human red cone opsin. **(a)** Time-dependent activation of transducin by human red cone opsin without (*open circles*) and with (*triangles*) 11-*cis*-retinal. At 5.5 min, the opsin with 11-*cis*-retinal was exposed to >530 nm light for 12 s demonstrating that pigment had formed and light-dependent activation occurred due to photoisomerization of the bound 11-*cis* form of the chromophore to the all-*trans* form. Note the reduction in transduction activation after 11-*cis*-retinal was added. The pH of the reaction was 6.4. **(b)** Relative transducin activation by expressed human red cone opsin after addition of 200 μ M retinal analogs [AT-RAL (all-*trans*-retinal); C17-RAL (17 carbon all-

trans-retinal analog); and β -ionone]. Activity was normalized to the activation by opsin alone.