11-*cis*- and All-*trans*-Retinols Can Activate Rod Opsin: Rational Design of the Visual Cycle[†]

Masahiro Kono,* Patrice W. Goletz, and Rosalie K. Crouch

Department of Ophthalmology, Medical University of South Carolina, Charleston, South Carolina 29425

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ABSTRACT: Rhodopsin is the photosensitive pigment in the rod photoreceptor cell. Upon absorption of a photon, the covalently bound 11-*cis*-retinal isomerizes to the all-*trans* form, enabling rhodopsin to activate transducin, its G protein. All-*trans*-retinal is then released from the protein and reduced to all-*trans*-retinol. It is subsequently transported to the retinal pigment epithelium where it is converted to 11-*cis*-retinol and oxidized to 11-*cis*-retinal before it is transported back to the photoreceptor to regenerate rhodopsin and complete the visual cycle. In this study, we have measured the effects of all-*trans*- and 11-*cis*-retinals and -retinols on the opsin's ability to activate transducin to ascertain their potentials for activating the signaling cascade. Only 11-*cis*-retinal acts as an inverse agonist to the opsin. All-*trans*-retinal, all-*trans*-retinol, and 11-*cis*-retinol are all agonists with all-*trans*-retinal being the most potent agonist and all-*trans*-retinol being the least potent. Taken as a whole, our study is consistent with the hypothesis that the steps in the visual cycle are optimized such that the rod can serve as a highly sensitive dim light receptor. All-*trans*-retinal is immediately reduced in the photoreceptor to prevent back reactions and to weaken its effectiveness as an agonist before it is transported out of the cell; oxidation of 11-*cis*-retinol occurs in the retinal pigment epithelium and not the rod photoreceptor cell because 11-*cis*-retinol can act as an agonist and activate the signaling cascade if it were to bind an opsin, effectively adapting the cell to light.

Rods are exquisitely sensitive to dim light. Rhodopsin is the photosensitive pigment that initiates the visual signaling cascade and is comprised of a protein part (opsin) and the 11-*cis* form of vitamin A aldehyde (11-*cis*-retinal) covalently bound to the opsin via a Schiff base linkage. Rhodopsin belongs to the superfamily of G protein-coupled receptors and uses the 11-*cis*-retinal not only as its chromophore but also as an inverse agonist, holding it in an inactive conformation (*1, 2*). Light converts the 11-*cis* bond to all-*trans*, changing the inverse agonist to an agonist to enable the opsin to activate its G protein transducin. In a competing reaction, rhodopsin kinase phosphorylates the light-activated rhodopsin, and arrestin binds to the phosporylated cytoplasmic surface to initiate the inactivation process. Eventually, rhodopsin is further deactivated when the chromophore is hydrolyzed from the opsin as all-*trans*-retinal, reduced to all-*trans*-retinol, and transported to the retinal pigment epithelium (RPE).[1] In the RPE, it is isomerized to 11-*cis*-retinol, oxidized to 11-*cis*-retinal, and then shuttled back to the photoreceptor cell to regenerate rhodopsin (Figure 1). The regeneration of rhodopsin whereby the retinoid is recycled through the RPE is called the visual cycle (*3*) [see also recent reviews (*4, 5*)].

Why would vertebrates evolve to include steps that convert an aldehyde to an alcohol for export and then import the reisomerized chromophore into the photoreceptor as an aldehyde? We address this question by examining the effects of 11-*cis*- and all-*trans*-vitamin A aldehydes and alcohols on the rod opsin's ability to activate transducin. Our results lead us to propose that the visual cycle is a process that helps to maximize the rod's sensitivity to dim light.

EXPERIMENTAL PROCEDURES

Materials. All-*trans*-retinal and -retinol were purchased from Sigma (St. Louis, MO). 11-*cis*-Retinal was synthesized as described previously (*6, 7*). 11-*cis*-Retinol was synthesized by reduction of 11-*cis*-retinal as described previously (*8*). The purity of retinoids was confirmed by high-performance liquid chromatography (*9*). Retinoid crystals were dissolved in ethanol and quantified by absorption spectroscopy using extinction coefficients as summarized previously (*10*). The rhodopsin 1D4 antibody was from National Cell Culture Center (Minneapolis, MN). Bovine retinas were from W. L. Lawson (Lincoln, NE). GTPγS-35 (1250 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Waltham, MA), and the nonradioactive GTPγS was from Roche Applied Science (Indianapolis, IN).

Protein Expression. Bovine rod opsin was expressed in COS cells as described previously (*11*). Cells were harvested 3 days after transfection and frozen at −80 °C. For membrane preparations, a discontinuous sucrose gradient was used to isolate the plasma membrane fraction (*12–14*). The amount of opsin in the membrane preparations was determined by

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* To whom correspondence should be addressed. E-mail: konom@musc.edu. Phone: (843) 792-6676. Fax: (843) 792-1723.

[1] Abbreviations: RPE, retinal pigment epithelium.

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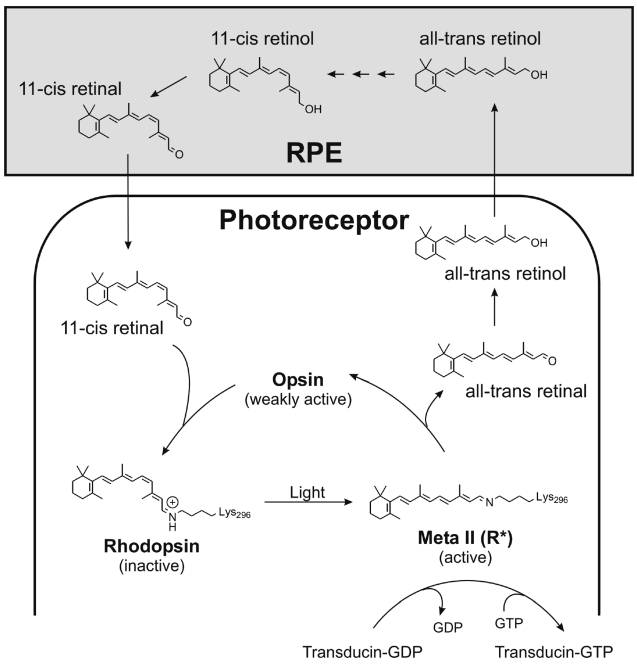

FIGURE 1: Schematic of the visual cycle. Photoactivation of rhodopsin isomerizes the 11-*cis* chromophore to the all-*trans* form, after which rhodopsin releases its chromophore, all-*trans*-retinal, which is reduced to all-*trans*-retinol. This is then transported to the RPE where it is converted to 11-*cis*-retinol via several steps, including esterification, isomerization, and hydrolysis by at least two enzymes (not shown) (e.g., see reviews in refs *4* and *5*). 11-*cis*-Retinol is oxidized to 11-*cis*-retinal in the RPE before it is transported back to the photoreceptor cell to bind an opsin to reform rhodopsin. The light-activated rhodopsin, Meta II, is the intermediate that activates transducin by catalyzing a GDP−GTP exchange.

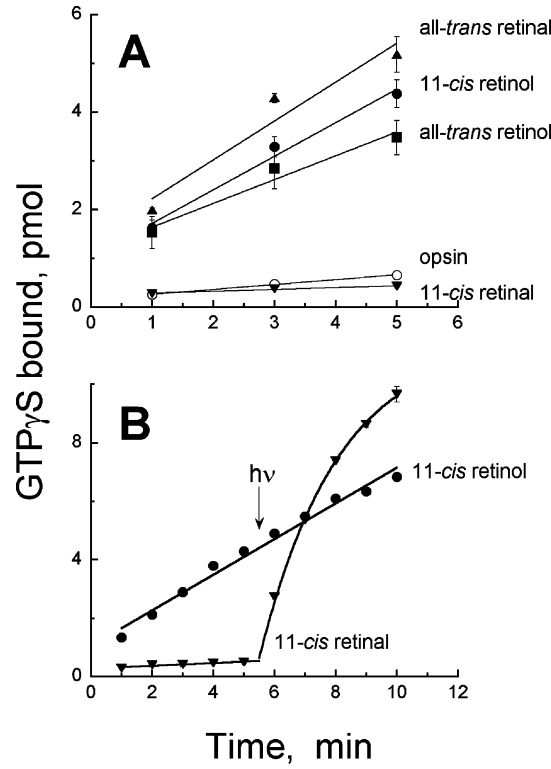

FIGURE 2: Activation of transducin by bovine rod opsin in the presence or absence of different retinoids and dark−light conditions. (A) Ligand-dependent transducin activation by rod opsin in the dark in the presence of all-*trans*-retinal (▲), 11-*cis*-retinol (●), all-*trans*-retinol (■), and 11-*cis*-retinal (▼). Also shown are data for the activation in the absence of ligand by the opsin itself (○). (B) Dark- and light-dependent activation of opsin incubated with 11-*cis*-retinal (▼) and 11-*cis*-retinol (●). At 5.5 min, samples were illuminated with >495 nm light for 12 s as described in Experimental Procedures.

slot blot analysis as described previously (*13*). Briefly, membrane preparation aliquots and known amounts rhodopsin from a bovine rod outer segment preparation were solubilized and loaded into the wells of the slot blot apparatus in triplicate. The blot was probed with the rhodopsin 1D4 antibody (*15*) and detected by chemiluminescence (*13*).

Transducin Activation Assay. Opsin activity was determined by its ability to activate bovine rod transducin using a radioactive filter binding assay essentially as described previously with a few modifications (*12, 13*). Bovine rod transducin was purified from bovine retinas as described previously (*16–18*). To assay the ability of the opsin to activate transducin, we followed the increase in the amount of radioactive GTPγS bound to transducin with time after addition of retinoid in the dark or after illumination. Typically, our reaction mixture contained 4 nM opsin (unless otherwise stated), 2.5 μM transducin, 3.0 μM GTPγS-35 (~5 nCi/μL), 20 μM retinoid, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.4), 100 mM NaCl, 1 mM dithiothreitol, and 5 mM $MgCl_2$ (pH 6.4) in a final volume of 50, 100, or 150 μL. The buffer, opsin membrane, and transducin were first mixed. Retinoids were added from a 50× stock dissolved in ethanol 1 min prior to addition of GTPγS, at which time the clock was started. At specified times, we transferred a 10 μL aliquot onto a nitrocellulose filter membrane attached to a vacuum manifold and washed it three times with 4 mL of ice-cold buffer; the amount of bound radioactivity was determined by scintillation counting. For assessing light-dependent activation, we began measuring the dark points, illuminated the sample with >495 nm light from a slide projector with a 300 W bulb passed through a long-pass filter for 12 s at a specified time, and then continued measuring 10 μL aliquots. We did not correct for the basal background activity of transducin itself at this lower pH or contributions from any endogenous COS cell receptors as they add little to the total activity and were shown previously to overlap with activity of rhodopsin in membranes in the dark (*1, 19*). Because ligand-dependent activation of transducin by opsins is linear, we also measured the amount of transducin activated at a single 5 min time point to quickly screen activation under a variety of conditions as described in the text and figure legends.

RESULTS

We tested the ability of bovine rod opsin to activate transducin in a ligand-dependent manner. Consistent with earlier results (*19*), 11-*cis*-retinal deactivates the opsin, which itself is weakly active (Figure 2A). The reaction pH used here is 6.4 because above neutral pH, the constitutive activity of opsin is difficult to resolve (*1*). In addition to increasing the relative activity of the opsin relative to rhodopsin in the dark, the lower pH also increases the basal activity of transducin itself (*1, 19*). We find that all-*trans*-retinal is an agonist to the rod opsin, increasing the activity 8-fold over opsin alone (Figure 2A). This result is consistent with earlier studies (*20–25*). Interestingly, the alcohols of the 11-*cis* and all-*trans* retinoids are also agonists, but they are not as robust

an as agonist as all-*trans*-retinal (5-fold over opsin alone for all-*trans*-retinol and 7-fold for 11-*cis* retinol) (Figure 2A).

To ensure that activity was not lowered due to oxidation of some 11-*cis*-retinol to retinal and formation of a pigment, we assayed for transducin activation by opsin incubated with 11-*cis*-retinol in the dark and after illumination with a 12 s pulse of >495 nm light. While this sample activated transducin at a rate higher than that of opsin alone, the activity did not change after exposure to light (Figure 2B), suggesting that a long-wavelength-absorbing pigment did not form with 11-*cis*-retinol. Illumination with ultraviolet light from a hand-held UV lamp also did not alter tranducin activation by opsin in the presence of 11-*cis*-retinol (not shown). Not surprisingly, the sample with 11-*cis*-retinal is able to activate transducin in a light-dependent manner because 11-*cis*-retinal formed a pigment absorbing maximally at 500 nm that can undergo *cis*−*trans* photoisomerization (Figure 2B). Under our conditions, the extent of light-dependent activation of transducin by rhodopsin is 30 times greater than that of transducin activated by opsin alone and 4 times greater than that by opsin with exogenously added all-*trans*-retinal.

The most effective agonist is the covalently bound all-*trans*-retinylidene formed from photoisomerization of the 11-*cis*-chromophore in rhodopsin. The next effective agonist is all-*trans*-retinal, followed by 11-*cis*-retinol and then all-*trans*-retinol. To ensure we were at a saturating level of retinoids, we assessed transducin activation by opsin incubated with varying concentrations of these agonists (Figure 3). All three agonists can activate the opsin in a concentration-dependent manner. The aldehyde is able to activate the opsin at much lower concentrations than the alcohols with an EC_{50} value of 53 nM for all-*trans*-retinal, while the EC_{50} values of the two alcohols are 6 μM. The reaction kinetics shown in Figure 2 were determined in the presence of 20 μM ligand. One striking feature of this result is the fact that the concentration dependence curves from the alcohols are steeper (fit with $n = 2$) than that from the aldehyde (fit with $n = 1$), suggesting cooperativity with the retinols.

We assessed transducin activation by doubling the amount of opsin membranes (opsin concentration, 8 nM) in the presence of different concentrations of all-*trans*-retinal and all-*trans*-retinol. When these data are normalized, they (white symbols, Figure 3A,B) are essentially indistinguishable from those collected with 4 nM opsin (black symbols, Figure 3A,B). We also show that the level of transducin activation by opsin alone and opsin in the presence of 20 μM all-*trans*-retinal increases linearly with increasing amounts of opsin (Figure 4). Thus, the opsin concentrations used in Figures 2 and 3 are in the linear range of activation.

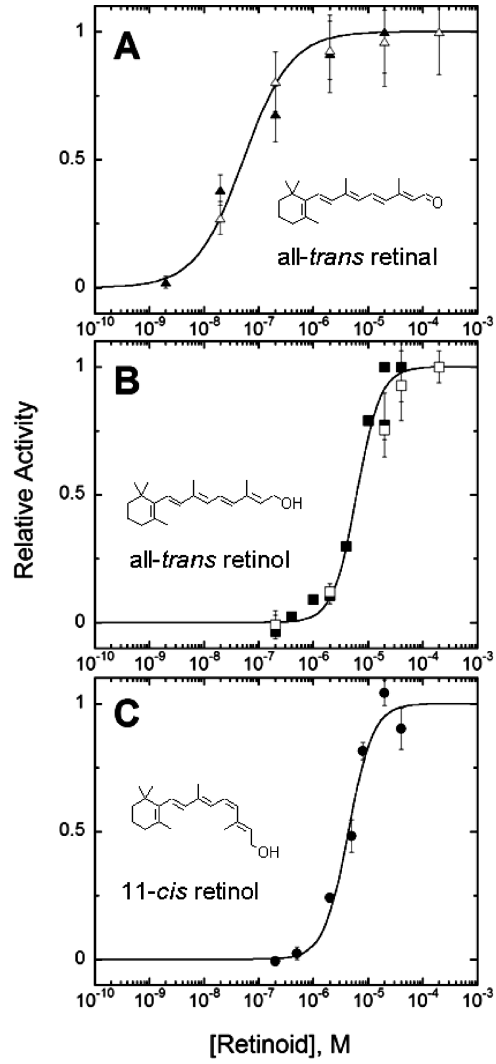

FIGURE 3: Agonist concentration dependence of transducin activation by rod opsin. The relative rates of transducin activation by 4 nM rod opsin (black symbols) are plotted vs different concentrations of (A) all-*trans*-retinal, (B) all-*trans*-retinol, and (C) 11-*cis*-retinol. The abilities of double the amount of opsin membranes (8 nM) to activate transducin in the presence of all-*trans*-retinal (A, △) or all-*trans*-retinol (B, □) are also shown.

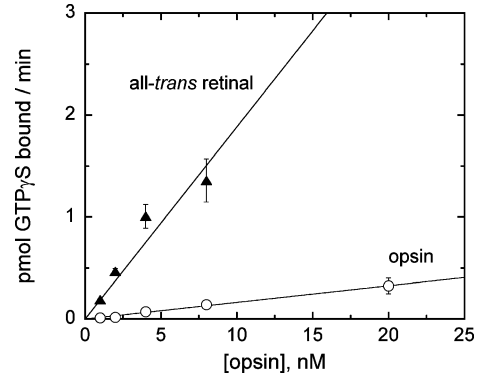

FIGURE 4: Activation of transducin as a function of opsin concentration in the absence (○) and presence of 20 μM all-*trans*-retinal. The reaction is performed as described in Experimental Procedures, but the amount of COS cell membranes containing the expressed opsin is varied.

DISCUSSION

Low opsin activity is necessary for rods to function under conditions of dim light. An opsin with a high basal level of activity would initiate the transduction cascade in the absence of light and desensitize the cells, compromising their high sensitivity. Such a mechanism is likely the cause for some forms of congenital night blindness. For example, the G90D rhodopsin mutant (*26*) opsin is constitutively active (*27*). A rod containing G90D rhodopsin seems to have a significant amount of apoprotein present in the dark (*28*), and its phenotype is that they appear as if they were light-adapted in the dark (*26, 29*).

Likewise, a prolonged presence of agonists in rods would be problematic because if they were able to bind an opsin, it would activate the signaling cascade and desensitize the rod in a manner independent of light. The first step in retinoid recycling after photoactivation of the pigment is release of all-*trans*-retinal from the opsin (*30, 31*). All-*trans*-retinal is then reduced to all-*trans*-retinol (*32*). There are a number of reasons why this step is advantageous to the photoreceptor cell. First, it eliminates the reassociation of an active complex with opsin (*33, 34*). Second, free all-*trans*-retinal is potentially toxic as both a visible light photosensitizer (*35*) and a source for A2E precursors (*36, 37*); all-*trans*-retinol is not. We now find that all-*trans*-retinol is an agonist to opsin, but it is a less potent agonist than all-*trans*-retinal in terms of a lower maximal amplitude of activation and the 2 order of magnitude difference in EC_{50} values. These would add additional levels of inactivation of the opsin on top of chromophore release and opsin phosphorylation and arrestin binding.

Exogenous all-*trans*-retinal and retinol have been shown not to compete with 11-*cis*-retinal for rhodopsin pigment regeneration (*21*). However, Schädel et al. (*38*) showed that these two all-*trans* retinoids do bind the rod opsin, suggesting that the binding site might differ from the site where 11-*cis*-retinal binds. An unexpected result in this study is the apparent cooperativity of the all-*trans*- and 11-*cis*-retinol concentration dependence curves that is not seen with all-*trans*-retinal (Figure 3). We have no current explanation for this result; however, it might be related to alternate retinoid binding sites in the opsin.

Previous studies as well as this one have shown all-*trans*-retinal to be a partial agonist (*20–25*), and we now show that all-*trans*-retinol can also be a partial agonist. Regardless of whether these retinoids are interacting with opsin at the same site as 11-*cis*-retinal or at a secondary site, the released retinal and its reduced product retinol need to be removed from the photoreceptor because otherwise their interaction with the opsin would continue to desensitize the rod cell. As mentioned above, reduction of all-*trans*-retinal to all-*trans*-retinol increases the EC_{50} from 53 nM to 6 μM, and this alone would lower the level of ligand-dependent activation of opsin. The upper limit of free retinoids in the absence of removal from the outer segment will be in the millimolar range because the rhodopsin concentration in the rod outer segment is 3 mM (*39*). This makes the concentration ranges of retinoids that activate opsin in this study physiologically possible after photobleaching rhodopsin and their removal helpful in limiting opsin activity especially under strong bleaching conditions.

Similarly, the oxidation of 11-*cis*-retinol outside of the rod photoreceptor and its subsequent transport back to the opsin as the aldehyde are advantageous because 11-*cis*-retinol is an even stronger agonist than all-*trans*-retinol. If 11-*cis*-retinol were to be transported to the rod cell, any opsin that were to interact with the retinol before its oxidation would be activated and consequently desensitize the rod. This action could explain the conclusion that 11-*cis*-retinol was "toxic" to bleached rods as described by Jones et al. (*8*) from studies on isolated salamander rods. Addition of 11-*cis*-retinol to bleached rods did not restore sensitivity and actually reduced sensitivity. Their results are consistent with rods not oxidizing 11-*cis*-retinol, and the 11-*cis*-retinol interacting with the rod opsin to activate the signaling cascade by activating transducin, which we show is possible in this study. However, the higher EC_{50} of the 11-*cis*-retinol concentration dependence data indicates that a significantly larger amount of 11-*cis*-retinol would be required for the rod opsin to activate transducin to the same level as with all-*trans*-retinal.

Among the retinoids in this study, only 11-*cis*-retinal behaves as an inverse agonist to rod opsin, and it is used to adapt the rod to dark as the rhodopsin pigment is generated. Photoisomerization of the bound ligand to the all-*trans* form activates the signal cascade. Hydrolysis of the chromophore and subsequent reduction to all-*trans*-retinol reduce the retinoid's efficacy as an agonist in addition to preventing the potential harmful effects of all-*trans*-retinal (*35–37*) from taking place. Its removal from the photoreceptor cell to the RPE prevents it from interacting with opsin. The generation of 11-*cis* retinol and its oxidation to 11-*cis*-retinal occur in the RPE, and the absence of 11-*cis*-retinol in the rod photoreceptor cell prevents the possibility of its interaction with opsin which would cause spurious activation of the signal cascade in the dark. Thus, the visual cycle appears to be exquisitely designed to optimize the rods' function as a dim light photoreceptor.

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