Activation of Transducin by a Xenopus Short Wavelength Visual Pigment

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Phototransduction in cones differs significantly from that in rods in sensitivity, kinetics, and recovery following exposure to light. The contribution that the visual pigment makes in determining the cone response was investigated biochemically by expressing a Xenopus violet cone opsin (VCOP) cDNA in COS1 cells and assaying the light-dependent activation of transducin. Light-exposed VCOP-stimulated \(^{35}S\)guanosine 5'-triphosphate nucleotide exchange on bovine rod transducin in a time-dependent manner with a half-time for activation of 0.75 min, similar to that of bovine rhodopsin. In exhaustive binding assays, VCOP and rhodopsin activity showed similar concentration dependence with half-maximal activation occurring at 0.02 mol of pigment/mol of transducin. Although VCOP was able to activate as many as 12 transducins per photoisomerization of cis-retinal, whereas a maximum of 1 transducin per VCOP was activated under brief illumination. The decay of the active species formed following photobleaching was complete in <5 min, ~10-fold faster than that of rhodopsin. In vitro, VCOP activated rod transducin with kinetics and affinity similar to those of rhodopsin, but the active conformation decayed more rapidly and the apoprotein regenerated more efficiently with VCOP than with rhodopsin. These properties of the violet pigment may account for much of the difference in response kinetics between rods and cones.

Photopic vision is mediated by specialized photoreceptor cone cells that function at high levels of illumination, respond to rapid changes in light, and permit color discrimination (1, 2). Each cone cell expresses a cone opsin. The cone opsins are members of a larger family of visual pigments (3–5) and share significant amino acid sequence homology with the rod pigment rhodopsin (6). Among the cone pigments, the short wavelength pigments (Group S, with wavelengths of peak absorbance \(\lambda_{max} \approx 415-440\) nm) permit vision in the violet/blue region of the spectrum and are represented by the mammalian blue, chicken violet, and Xenopus violet pigments.² Although cone cells expressing Group S pigments are in the minority in the vertebrate retina, they are an integral part of vision. For example, in blue monochromats, i.e. humans with red-green pigment mutations, the blue opsin is the sole mediator of photopic vision (7, 8).

The phototransduction mechanisms have been extensively investigated in rods (for reviews see Refs. 2 and 9). Following absorption of light and isomerization of 11-cis-retinal to all-trans-retinal, rhodopsin undergoes a series of conformational changes that eventually leads to a transient state, coincident with metarhodopsin(II) (MetaII), that activates the second messenger cascade (9). MetaII interacts with the heterotrimeric rod G protein, transducin, and thereby catalyzes the exchange of guanyl nucleotide, which leads to dissociation of the transducin subunits. Rods and cones express a very similar set of transduction proteins (10); however, in general, the electrical response of a cone to light is faster and less sensitive than that of a rod (11, 12). Moreover, there are differences in the properties of responses even between the various cone subtypes. In salamanders, responses of violet cones to light exhibit more rod-like behavior, with slower kinetics and increased sensitivity than long wavelength (red) cones (11). The molecular basis for the differences between cone and rod responses and among the cone subtypes has not been established.

In order to investigate the phototransduction properties of the short wavelength visual pigments, a Xenopus violet cone opsin (VCOP) was cloned and characterized.² The protein, which was efficiently expressed in COS1 cells, formed a Schiff base with 11-cis-retinal and absorbed maximally at 425 nm. Characterization of the light-dependent interaction of VCOP with transducin was performed to identify the contribution of the pigment to the physiological responses of cones. Using a guanyl nucleotide exchange assay and purified bovine rod transducin, we found that VCOP activated rod transducin with kinetics and affinity similar to those of rhodopsin but that the active conformation decayed more rapidly and the apoprotein regenerated more efficiently than rhodopsin.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Visual Pigments—An expression construct, pMT-VCOP, containing the first 328 codons of the Xenopus violet cone opsin coding region and the last 14 codons of bovine rod opsin was expressed in mammalian COS1 cells by transient transfection (13). The

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² The abbreviations used are: \(\lambda_{max}\), wavelengths of peak absorbance; MetaII, metarhodopsin(II); VCOP, violet cone opsin; V*, photoexcited violet cone pigment; V-MetaII, violet cone metarhodopsin(II)-like photocmediate DMS; CTPS, guanosine 5'-triphosphate; ROS, rod outer segment; ADH, alcohol dehydrogenase.

² D. M. Starace and B. E. Knox, submitted for publication.
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epitope-tagged violet cone pigment (VCOP) was generated by incubation with 11-cis-retinal and purified in buffer W (50 mM HEPES, pH 6.6, 140 mM NaCl, 3 mM MgCl₂, 20% (w/v) glycerol, and 0.1% dodecyl maltoside (DM)), as described elsewhere. Xenopus rhodopsin, in the construct pMT-XOP1 (14), was similarly expressed and purified. Bovine rhodopsin from rod outer segments (ROS) was purified in parallel with the expressed pigments. UV-visible absorption spectra of purified and solubilized visual pigments were recorded in the indicated buffers at 20°C for varioustimes, and then added into the transducin assay for a 30-min reaction. Samples that were exposed to light prior to transducin assay were illuminated for 1 min, incubated in the dark. The lines represent single parameter exponential fits, with half-times for activation of 0.75 min for Xenopus violet pigment, 1.0 min for Xenopus rhodopsin, and 0.55 min for bovine rhodopsin. B. titration of rod transducin activation by the Xenopus violet pigment. Various amounts of Xenopus violet pigment (A) or bovine ROS rhodopsin (B) were incubated with 60 pmol of bovine rod transducin in 0.0075% dodecyl maltoside in volumes of 200 µl. Reactions proceeded for 30 min under continuous illumination (λ > 420 nm). The solid lines are sigmoidal fits to the data using the Hill equation with 1.2 pmol (6 nM) of pigment required to activate 50% of the transducin for both samples.

RESULTS

The Xenopus Violet Cone Pigment Activates Transducin—In order to examine the interaction between short wavelength visual pigments and transducin, the violet opsin was expressed in COS1 cells containing a carboxyl-terminal 1D4 epitope tag from bovine rhodopsin (VCOP). Visual pigment was generated in COS1 membranes by incubation with 11-cis-retinal, solubilized in DM, and purified by immunoaffinity chromatography on 1D4-Sepharose. The presence of the altered carboxyl terminus in the expressed VCOP was not expected to perturb the transducin activity significantly since the carboxyl domain does not appear to play an important role in transducin activation by rhodopsin (17, 18). The low abundance of transducin from Xenopus or bovine retina, precluded the use of bovine transducin in the biochemical assays. However, the high degree of homology between VCOP and bovine rhodopsin in the regions shown to be involved in the interaction of light-activated rhodopsin with rod transducin (17, 19) and the similarity of rod and cone transducin α subunits in mammals (10) and in Xenopus suggested that VCOP would interact with rod transducin.

VCOP activated bovine rod transducin in a time-dependent manner (Fig. 1A) with a half-time of activation (t1/2) of 0.75 min. The reaction was complete in 5 min and resulted in only 24% of the available transducin being activated. Both COS1-expressed Xenopus rhodopsin and bovine ROS rhodopsin were able to completely activate transducin (Fig. 1A). Thus, although a single rhodopsin was able to catalyze the activation of multiple transducins, a single VCOP maximally stimulated only one transducin. The kinetics of activation of the rhodopsins were similar to those of VCOP (t1/2 = 0.58 and 1.0 min for Xenopus and bovine rhodopsin, respectively), suggesting that the rate of interaction between transducin and the active state of VCOP is similar to that of the rhodopsins. However, the initial rate of activation was slower for VCOP (0.46 mol of GTP·S bound/mol of opsin/min) than for Xenopus (1.44 mol of GTP·S bound/mol of opsin/min) and bovine rhodopsin (2.1 mol of GTP·S bound/mol of opsin/min).

In order to determine the total number of interactions that occur between VCOP and transducin, a second set of experiments was performed with a fixed concentration of rod transducin, VCOP caused a concentration-dependent stimulation of nucleotide exchange on transducin (Fig. 1B) with a sigmoidal behavior and half-maximal activity occurring at 1.2 pmol of pigment. Bovine ROS rhodopsin similarly stimulated transducin with a half-maximal activity also occurring at 1.2 pmol of bovine rhodopsin (Fig. 1B). Thus, the apparent affinity between VCOP and rod transducin was similar to that observed with

3 B. E. Knox, unpublished data.
rhodopsin. However, the maximum level of activity of VCOP occurred at 21 pmol of GTP·S bound, indicating that only 35% of available transducin was activated, in contrast to bovine rhodopsin, which activated all available transducin. The incomplete activation of transducin by VCOP was not due to the inability of a single VCOP molecule to activate more than one transducin, since multiple turnovers were observed at lower pigment concentrations (e.g. 1.2 pmol of pigment catalyzed the exchange of 14 pmol of GTP·S). By contrast, bovine rhodopsin stimulated the turnover of up to 36 transducins at its half-maximal concentration (Fig. 1B) in agreement with previous studies of the activity of bovine rhodopsin in detergent (20). The similarity of the initial rate values of VCOP and rhodopsin and the identical concentration dependence for activation of transducin suggest that the interaction of the active state (V-MetaII or V*) of VCOP with rod transducin has a rate and an affinity comparable to those of rhodopsin.

The failure of VCOP to completely activate transducin was not due to a decay of the pigment during the incubation period of the transducin assay since the activity of VCOP preincubated for 30 min at room temperature before addition of transducin was equal to the activity without preincubation (data not shown). The hydrophobic media, i.e. lipid and detergent, in which transducin reactions occur play an important role in stabilizing activity. The interaction between bovine rod transducin and VCOP was measured in 0.007–0.01% DM since previous work has demonstrated that this detergent concentration range permits optimal enzymatic activity of solubilized bovine rhodopsin (20). The optimal concentration of DM for VCOP activity sharply peaked at 0.008% (Fig. 2), which is near the critical micelle concentration of the detergent (21). The detergent profiles of bovine and expressed *Xenopus* rhodopsin activities were the same as that of VCOP (Ref. 20 and data not shown). Thus, the incomplete activation of bovine rod transducin by VCOP at saturation cannot be attributed to nonoptimal detergent conditions.

To directly demonstrate that the light intensity (λ > 420 nm) was not limiting in transducin binding assays, bleaching of VCOP was monitored spectroscopically under conditions identical to assay conditions except that transducin was omitted (Fig. 3, left panel). Photoactivation of VCOP caused a decay of the pigment (λ<sub>max</sub> 425 nm) and a concurrent formation of a product with λ<sub>max</sub> ~ 375 nm. This product could be free all-trans-retinal, which is released from the pigment upon bleaching, or V-MetaII, which is expected to be spectrally indistinguishable from free retinal. Acid denaturation experiments have shown that the product is most likely free all-trans-retinal (data not shown). A dark-light difference spectra (Fig. 3B) more clearly shows the progress of pigment bleaching, since the absorbances of VCOP and the photoproducts significantly overlap. After 10 s of illumination, >85% of the 425 nm absorbance has been converted. In addition, there was an increase in light scattering at shorter wavelengths, indicating that VCOP aggregates on bleaching. After 20 s of illumination, there was no additional change in the 425 nm peak, indicating that 20 s is enough time to bleach VCOP completely. The spectroscopic analysis of VCOP bleaching provides direct evidence that, under the transducin assay conditions, the VCOP was sufficiently photostimulated and cannot account for the failure of VCOP to activate all of the available transducin.

**Decay of the Active State**—The ability of VCOP to activate rod transducin suggests that illumination creates a metastable active state analogous to MetaII. To further characterize the interaction between VCOP and transducin, the lifetime of the photoexcited state (V*) was measured. V* was formed by brief illumination of VCOP and its lifetime was monitored by addi-
of transducin and [3S]GTPγS for nucleotide exchange assay at various times after illumination. The light-activated VCOP exhibited a rapid decay of its ability to stimulate GTPγS exchange. The half-time of decay was approximately 2 min and a complete loss of activity occurred in 5 min (Fig. 4). In contrast, light-activated rhodopsin exhibited more prolonged activity with a half-time of decay of 20 min and detectable activity even after 2 h. Thus, the reduced activity of VCOP when compared to that of rhodopsin is attributable to the relatively reduced lifetime of its photoactive state and not to a reduced rate of interaction with or affinity for rod transducin.

Efficient Photoregeneration of Violet Cone Opsin—Unlike rhodopsin (data not shown), transducin activation by VCOP under these in vitro conditions depended on the illumination conditions during the assay (Fig. 5). Using λ > 420 nm light, there was an approximately linear increase in the activity observed with the illumination time, showing an almost 10-fold increased activity with 30 min of illumination compared to 1 min. The activity of VCOP was also measured as a function of the spectral composition of the saturating illumination used during the transducin assay in reactions continuously exposed to one of the following illumination conditions: λ > 455 nm, λ > 440 nm, λ > 420 nm, or unfiltered projector light (white light) (Fig. 6). Maximal and equivalent activities were observed when VCOP was illuminated for 30 min with white light or with λ > 420 nm light. Illumination of the pigment for 30 min with λ > 440 nm or λ > 455 nm filtered light resulted in increasingly reduced activities even though VCOP was bleached by these lighting conditions.
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FIG. 7. The *Xenopus* violet pigment activity is sensitive to horse liver alcohol dehydrogenase. *Xenopus* violet pigment (2 pmol) was incubated with 60 pmol of bovine rod transducin and [35S]GTPγS in 200 µl reactions containing 0.007% dodecyl maltoside. Reactions were performed in the presence (solid bars) or absence (open bars) of excess horse liver alcohol dehydrogenase (20 milliunits) and NADH (175 µM). Reactions were illuminated (λ > 420 nm) for the indicated times and terminated after 30 min for measurement of bound nucleotide.

The photoisomerization of all-trans-retinal to an equilibrium mixture of isomers, including 11-cis-retinal, is produced with high intensity near-UV light in aqueous solutions (22, 23). The recycling of released and photoisomerized all-trans-retinal chromophore by subsequent regeneration of VCOP apoprotein is consistent with the observed increase of activity with illumination (Fig. 5) and with the spectral profile of activity (Fig. 6). In order to determine whether pigment recycling was occurring, activation of transducin by VCOP was carried out in the presence and absence of horse liver alcohol dehydrogenase (ADH) and its cofactor, NADH (Fig. 7). In the presence of excess NADH at pH 7.0, ADH reduces retinal to retinol (24), thereby preventing the regeneration of visual pigment by recycling of the chromophore. The increase of VCOP activity with the time of illumination was eliminated when excess ADH and NADH were added, whereas the activity for the brief illumination was not changed. The sensitivity to ADH and NADH provides evidence that the illumination time-dependent VCOP activity is due to the photoisomerization and recycling of released chromophore.

In another set of experiments, the regeneration of VCOP with recycled chromophore was explicitly monitored by measuring the ADH-sensitive transducin activity of bleached VCOP. Before addition of rod transducin, VCOP was illuminated (λ > 420 nm) for 30 s and preincubated for 30 min in the dark at room temperature to allow complete decay of the resulting active state. In addition, the dark incubation was carried out in the presence and absence of ADH and NADH and/or 11-cis-retinal. Upon addition of transducin to the bleached VCOP, the reaction proceeded for 30 min in the dark or under constant illumination (λ > 420 nm) to assay illumination-dependent activity (Fig. 8). The bleached VCOP showed light-sensitive activity. There was no activity when the assay was performed in the dark. The light-sensitive activity of the bleached VCOP was severely inhibited by the addition of excess ADH and NADH. This result shows that the *Xenopus* violet opsin protein rebinds 11-cis-retinal, formed by photoisomerization of the released chromophore, to regenerate an active pigment. The addition of 1–2 molar excess over pigment of exogenous 11-cis-retinal during the dark preincubation, immediately after preillumination, enhanced the light-sensitive activity of the bleached pigment by 1.7-fold, and this activity was also sensitive to ADH and NADH. The increase of activity caused by illumination, despite substoichiometric formation of 11-cis-retinal by photoisomerization, suggests that the affinity of *Xenopus* violet opsin for 11-cis-retinal is quite high.

**FIG. 8.** The activity of bleached violet pigment is dependent on illumination and ADH. *Xenopus* violet pigment (2 pmol) was incubated with 60 pmol of bovine rod transducin and [35S]GTPγS in 200 µl reactions containing 0.007% dodecyl maltoside. Bleached violet pigment was prepared at room temperature by preillumination (λ > 420 nm) the reaction mixture without transducin for 30 s followed by a 30-min preincubation in the dark. When indicated, ADH and NADH were added. During the dark preincubation, 11-cis-retinal (2–4 pmol) was added immediately after the preillumination. Bovine rod transducin was added and the reaction proceeded for 30 min in the dark (solid bar) or with continuous illumination (λ > 420 nm) (open bars).

**DISCUSSION**

Despite the similarities in the components of the cone and rod visual transduction cascade, the physiological responses of cone photoreceptor cells differ from those of rods in a number of ways (11, 12). First, cones are less sensitive than rods; 100 photons are required to elicit an electrical response of a cone cell, as opposed to 1 photon for a rod cell. Second, the kinetics of the electrical response of a cone cell have a rate 3–10-fold faster than that of the rod cell. Finally, the rate of regeneration of a cone is 500 times faster than that of a rod. However, the biochemical and molecular mechanisms that underlie these phenomena are unclear, largely due to the low abundance and heterogeneity of cone phototransduction components in most retinas. Using methods for mammalian cell expression of opsins, the purified violet cone visual pigment was generated in quantities amenable to a detailed study of its interaction with rod transducin, which can be purified in abundance and, is homologous to cone transducin (10). Although the rates and extents of activity measured using rod transducin may not be the same for the activation of cone-transducin, the observed activity is proportional to the amount of V* formed. The characterization of the interaction of the violet pigment and rod transducin permitted the investigation of the role of the visual pigment itself in the response properties of the cone cell.

The *Xenopus* violet pigment was able to activate rod transducin in both a concentration- and a time-dependent manner. The amount of pigment required to stimulate half-maximal activity was the same as the half-maximal value for bo-
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These experiments demonstrate the effectiveness of using expressed *Xenopus* violet cone opsins as a model system for exploring cone visual pigment biochemistry and structure-function relationships. Previously, several studies on the chicken red cone pigment have demonstrated that it activates transducin (4, 25, 27) and the cGMP phosphodiesterase (28) and is a substrate for rhodopsin kinase (29). Although these studies have demonstrated the basic interactions, kinetic comparisons relevant to cone-rod physiological differences were not carried out. The experiments reported here have extended the biochemistry of phototransduction to the cone pigments and are novel for the short wavelength group of cone pigments. Recent spectroscopic work on chicken green opsin and its photobleaching intermediates (26, 30) have postulated differences in signal transduction properties between rod and cone pigments arising from differences in the MetaII lifetimes. The results presented in this report have confirmed this idea. Future work will be directed toward identifying the molecular basis for the different phototransduction properties exhibited by the short wavelength visual pigment and rhodopsin.

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