Probing the Catalytic Sites and Activation Mechanism of Photoreceptor Phosphodiesterase Using Radiolabeled Phosphodiesterase Inhibitors*

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Retinal photoreceptor phosphodiesterase (PDE6) is unique among the phosphodiesterase enzyme family not only for its catalytic heterodimer but also for its regulatory γ-subunits (Pγ) whose inhibitory action is released upon binding to the G-protein transducin. It is generally assumed that during visual excitation both catalytic sites are relieved of Pγ inhibition upon binding of two activated transducin molecules. Because PDE6 shares structural and pharmacological similarities with PDE5, we utilized radiolabeled PDE5 inhibitors to probe the catalytic sites of PDE6. The membrane filtration assay we used to quantify [3H]vardenafil binding to PDE6 required histone II-AS to stabilize drug binding to the active site. Under these conditions, [3H]vardenafil binds stoichiometrically to both the α- and β-subunits of the activated PDE6 heterodimer. [3H]vardenafil fails to bind to either the PDE6 holoenzyme or the PDE6 catalytic dimer reconstituted with Pγ, consistent with Pγ blocking access to the drug-binding sites. Following transducin activation of membrane-associated PDE6 holoenzyme, [3H]vardenafil binding increases in proportion to the extent of PDE6 activation. Both [3H]vardenafil binding and hydrolytic activity of transducin-activated PDE6 fail to exceed 50% of the value for the PDE6 catalytic dimer. However, adding a 1000-fold excess of activated transducin can stimulate the hydrolytic activity of PDE6 to its maximum extent. These results demonstrate that both subunits of the PDE6 heterodimer are able to bind ligands to the enzyme active site. Furthermore, transducin relieves Pγ inhibition of PDE6 in a biphasic manner, with only one-half of the maximum PDE6 activity efficiently attained during visual excitation.

The superfamily of phosphodiesterase (PDE) enzymes plays a critical role in maintaining the cellular levels of cAMP and cGMP (1). Photoreceptor phosphodiesterase (PDE6) is the central effector responsible for lowering cGMP levels in photoreceptor cells following light stimulation. The PDE6 activation mechanism, its catalytic efficiency, and its substrate specificity are all designed to optimize the ability of photoreceptors to rapidly respond to light stimuli with subsecond changes in cGMP levels (2). During the first steps in vision, photoisomerized rhodopsin activates transducin, which binds GTP and releases its activated α-subunit (Tα-GTP) to activate membrane-associated rod PDE holoenzyme by displacing the inhibitory γ-subunit (Pγ) from the active sites of the PDE6 catalytic dimer (Pαβ). The drop in cGMP that results from PDE6 activation causes cGMP-gated ion channels to close, resulting in membrane hyperpolarization that is transmitted to second order retinal neurons (3, 4).

Considering the wealth of quantitative information about the phototransduction pathway, it is surprising that important aspects of PDE6 function and regulation remain unknown. For example, rod PDE6 usually exists as a tightly associated catalytic dimer of α- and β-subunits (Pαβ), but there are still questions about whether one or both of the catalytic domains are active. Underscoring this point is the fact that chicken rod photoreceptor PDE6 apparently contains only one functional catalytic subunit (β-subunit) (5), raising the possibility that the catalytic site on the α-subunit in other species is not functional. Moreover, there is no consensus in the literature on the issue of whether transducin can fully activate PDE6 catalysis. Although it has been assumed that transducin can activate PDE6 in a 1:1 molar ratio (6, 7), the question of whether one or both PDE6 catalytic sites become activated by transducin during visual excitation has never been demonstrated. In some instances, it has been reported that two Tα-GTP bind to both catalytic subunits of Pαβ releasing the Pγ inhibition at both active sites (6, 8). Other investigators have reported that a single Tα-GTP was able to maximally activate the PDE6 catalytic dimer under defined conditions (9–11). The latter work suggests that either the PDE6 catalytic dimer has only one functional active site or that a single activated Tα-GTP can relieve Pγ inhibition at both Pαβ active sites. Furthermore, it is reported that transducin can activate PDE6 to approximately one-half of the rate that is seen if the γ-subunits are physically removed from PDE6 in frog (12, 13) and bovine (10, 14) rod outer segments. This has led to conflicting models of transducin activation of PDE6 in which transducin is hypothesized to relieve Pγ inhibition at either one or both catalytic sites of PDE6.
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PDE6 differs in several fundamental ways from the other 10 classes of mammalian phosphodiesterases. Rod PDE6 is the only PDE that exists as a catalytic heterodimer, whereas cone PDE6 and the other 10 PDE families are all believed to be homodimers. Unlike other PDE families, rod and cone PDE6 catalytic activity is primarily regulated by distinct inhibitory Pγ subunits tightly associated with the catalytic dimer to form an inactive tetrameric holoenzyme (15). PDE6 is also the only family of PDEs in which the catalytic activity is directly regulated by a heterotrimeric G-protein, transducin (2).

PDE6 is most closely related to PDE5 (abundant in vascular smooth muscle) in its biochemical, structural, and pharmacological properties (16). Both PDE5 and PDE6 have highly conserved amino acid sequences and three-dimensional structures (17–20). PDE5 and PDE6 share strong substrate specificity for cGMP compared with cAMP (21). Both can bind cGMP with high affinity at one of their regulatory GAF domains within each catalytic subunit (2, 22). Most PDE5-selective inhibitors, including the well known erectile dysfunction drugs Viagra (sildenafil) and Levitra (vardenafil), can also potently inhibit PDE6 catalysis as well (23–25).

We used the ability of PDE5 inhibitors to bind with high affinity to PDE6 to probe the active sites of the enzyme and to better elucidate the activation mechanism by transducin. Using [3H]vardenafil, we tested the hypothesis that both catalytic domains of the Pαβdimer are catalytically active and functionally equivalent. We then evaluated whether binding of activated transducin to the PDE6 holoenzyme relieves inhibition at one or both of the active sites in the PDE6 dimer.

EXPERIMENTAL PROCEDURES

Materials—Bovine retinas were purchased from W. L. Lawson, Inc. Superdex 200 and Mono-Q columns were from GE Healthcare, Inc., and the C18 reversed phase column (300A, 22 × 250 mm) was from Vydac. Filtration and ultrafiltration products were from Millipore. Scintillation fluid (Ultima Gold-XR) and [3H]cGMP were from PerkinElmer Life Sciences, and [3H]vardenafil (26) was a kind gift of Drs. P. Sandner, E. Bischoff, and U. Pleiss (Bayer Healthcare AG). Protein assay reagents were from Pierce, and all other chemicals were obtained from Sigma.

Preparation of Bovine Rod Outer Segments (ROS), PDE Holoenzyme, and PDE Heterodimer—Bovine ROS were prepared from frozen bovine retinas under dark-adapted conditions on a discontinuous sucrose gradient (27). Rod PDE6 holoenzyme (Pαβγγ) was extracted with a hypotonic buffer from illuminated ROS homogenates and purified by Mono-Q anion exchange chromatography and Superdex 200 gel filtration chromatography. The purified PDE6 (≥95% pure) was then concentrated by ultrafiltration and stored with 50% glycerol at −20 °C (27).

The PDE6 catalytic dimer (Pαβ) was prepared from the PDE6 holoenzyme by removing the inhibitory Pγ subunits through limited trypsin proteolysis (28). A time course of proteolytic activation of PDE6 was determined to ensure that >90% of the Pγ subunit was destroyed without altering the apparent molecular weight of the catalytic subunits (as judged by SDS-PAGE) (27).

The concentration of PDE6 was determined by both measurements of catalytic activity under conditions where the kcat was known (5600 s⁻¹) (29) and by measurements of [3H]cGMP binding under nucleotide-depleted conditions (described in Ref. 30) where the cGMP-binding sites in the GAF domains were unoccupied and stoichiometric binding (2.0 cGMP/PDE6 dimer) occurred (31).

Purification of Persistently Activated Transducin α-Subunit (Tα-GTPγS)—Transducin α-subunits were extracted from the PDE6-depleted ROS membranes by adding 50 μM GTPγS (in low salt buffer) to the ROS membranes and recovering the solubilized Tα-GTPγS by centrifugation. The extracted Tα-GTPγS was purified on a Blue Sepharose column (32, 33). The concentration of Tα-GTPγS was determined by a colorimetric protein assay. Purified Tα-GTPγS was stored with 50 μM GTPγS and 50% glycerol at −20 °C.

Preparation and Purification of Tγ and a C-terminal Synthetic Peptide—Wild-type bovine rod Pγ (87 amino acids) was expressed in Escherichia coli BL21(DE3) cells and purified to >97% purity using SP-Sepharose followed by reversed phase high pressure liquid chromatography (34). The wild-type Pγ concentration was determined spectrophotometrically using an experimentally determined extinction coefficient of 7550 cm⁻¹ M⁻¹ (31). The inhibitory activity of purified Pγ was assayed by its ability to stoichiometrically inhibit trypsin-activated bovine rod PDE (29). The spectrophotometric and activity estimates of Pγ concentration agree to within 10% for all of the wild-type Pγ preparations used in this study. The concentration of the synthetic peptide Pγ63–87 (New England Peptide, Inc.) was determined by a protein assay.

Transducin Activation of ROS PDE—Purified ROS were resuspended in buffer A (20 mM MOPS, 2 mM MgCl₂, 30 mM KCl, 120 mM NaCl, pH 7.4) at a concentration of 30 μM rhodopsin and then passed through a 26-gauge insulin needle 10 times under dim red light. The concentration of membrane-associated PDE was estimated based on its stoichiometric ratio to rhodopsin (300 rhodopsin/PDE6) and its maximum hydrolytic activity after trypsin proteolysis. After ROS homogenates were fully bleached by light to activate rhodopsin, PDE6 was activated by incubation with an excess of GTPγS relative to the transducin concentration; the rate of cGMP hydrolysis was then assayed.

Binding of [3H]Vardenafil to Catalytic Sites on PDE—The membrane filtration assay to quantitate [3H]vardenafil binding to PDE6 was adapted from a similar assay for PDE5 (35). The standard binding assay buffer contained histone Type II-AS (0.2 mg/ml). To reduce nonspecific binding, the samples were diluted 20-fold with ice-cold wash buffer (10 mM Tris, pH 7.5, 0.1% Triton X-100) immediately before applying the sample onto pre-wet Millipore HAWP 025 membrane filters. The filters were washed eight times with 1 ml of ice-cold wash buffer.

Analytical Methods—The rate of cGMP hydrolysis was determined by a phosphate release assay (31). Activity measurements were made in 100 mM Tris (pH 7.5) buffer containing 10 mM MgCl₂, 0.5 mg/ml bovine serum albumin, 0.5 mM EDTA, 2 mM dithiothreitol. All of the rate measurements were obtained from four individual time points at saturating cGMP concentrations (10 μM), and less than 30% substrate was con-
assumed during this time. The [3H]cGMP membrane filtration binding assay was used to determine the stoichiometry of cGMP binding under various conditions (36) with 10 mM EDTA and 50 μM vardenafil added to the binding assay solution to prevent cGMP hydrolysis. (Note that vardenafil did not alter cGMP binding, consistent with previous work demonstrating the very low affinity of PDE inhibitors or most cGMP analogs to occupy the cGMP-binding sites of the PDE6 GAF domains (24, 37).) The rhodopsin concentration was spectrophotometrically determined, using an extinction coefficient of 42,000 M⁻¹ cm⁻¹ (38). Protein concentrations were determined by the bicinchoninic acid protein assay (39) using bovine γ-globulin as a standard. Curve fitting and statistical analyses were carried out with Sigmaplot. Unless otherwise noted, all of the experiments were performed three times.

RESULTS AND DISCUSSION

Histone II-AS Stabilizes [3H]Vardenafil Binding to the Catalytic Sites of PDE6 Catalytic Dimer—Previous work has shown that most of the so-called PDE5-selective inhibitors (e.g. zaprinast, E4021, sildenafil, and vardenafil) also inhibit the catalytic activity of the closely related photoreceptor PDE6 (23–25). To date, vardenafil is the most potent of this class of catalytic site inhibitor, with an inhibition constant for PDE6 of ~1 nM (25). As such, it represents a useful tool for probing the active sites of PDE6 in its nonactivated and activated states. Initial experiments measuring [3H]vardenafil binding to purified PDE6 using a membrane filtration assay revealed high nonspecific binding and variability in total binding. There was no detected binding of [3H]vardenafil to Pαβ alone. Consistent with previous studies of PDE5 binding to radiolabeled inhibitors (35), we observed that histone II-AS stabilized [3H]vardenafil binding to PDE6 catalytic dimer (Pαβ) in a concentration-dependent manner (Fig. 1). Interestingly, histone II-AS had little effect on cGMP hydrolytic activity, Pγ binding affinity, or the ability of [3H]cGMP to bind to the regulatory GAF domains (data not shown). The mechanism of histone II-AS effects on PDE5 or PDE6 is still not clear, but one likely possibility is that histone II-AS slows down drug dissociation during the washing step of the filter binding assay.

To further characterize the unexpected stabilization of [3H]vardenafil binding by histone II-AS, we tested several other known PDE6-interacting compounds, including histone Type VIII-S (40, 41), Pγ, and Pγ peptides. Histone VIII-S, which historically has been used to displace Pγ and activate the PDE6 holoenzyme, can stabilize [3H]vardenafil binding to the Pαβ dimer to a certain extent (Fig. 2) but did not reach the maximum binding observed with histone II-AS. Little [3H]vardenafil binding was detected with Pγ or the C-terminal peptide Py63-87, as might be expected because the C-terminal region of the inhibitory Pγ subunits competes with drug binding at the active sites (25, 42).

[3H]Vardenafil Binds to PDE6 Catalytic Dimer, but Pγ Blocks Inhibitor Binding to the Holoenzyme—It is well established that rod PDE6 consists of α and β catalytic subunits, and it has been assumed, but never demonstrated, that each catalytic domain of Pαβ is active. The binding assay for [3H]vardenafil allowed us to directly test this by comparing catalytic activity, cGMP binding stoichiometry, and [3H]vardenafil binding in the same Pαβ catalytic dimer preparation.

Fig. 3 shows that the binding curve for [3H]vardenafil to purified Pαβ displays a single class of drug-binding sites. The apparent binding affinity for vardenafil is high but lower than the reported value of the inhibition constant (Kᵢ = 0.7 nM) (25). This discrepancy may be due to the requirement for a nanomolar level of PDE6 to reproducibly quantify [3H]vardenafil binding, resulting in titration of the binding site as the vardenafil concentration is increased. This interpretation is supported by
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FIGURE 3. $[^3]H$Vardenafil binds stoichiometrically to each $\alpha\beta$ catalytic subunit, but only in the absence of Py. Purified $\alpha\beta$ (2.5 nm; filled circles) or $\alpha\beta$ reconstituted with 10 µM Py (open circles) was incubated with 0.2 mg/ml histone II-A5 and the indicated concentration of $[^3]H$Vardenafil. The samples were incubated for 40 min before membrane filtration. Vardenafil binding was normalized to the $\alpha\beta$ concentration, as estimated by both $[^3]H$cGMP binding assay, as well as hydrolytic activity measurements (which agreed to within 10%). The solid line represents the fit of the data for $[^3]H$vardenafil binding to $\alpha\beta$ assuming a single class of binding sites (apparent $K_d = 7.8$ nm and $B_{\text{max}} = 2.0$). The data are representative of three similar experiments.

experiments in which the apparent $K_d$ for $[^3]H$vardenafil binding decreased as the PDE6 concentration was lowered (data not shown). The maximum extent of $[^3]H$vardenafil binding to purified $\alpha\beta$ was calculated to be $2.1 \pm 0.1$ (S.D.; $n = 4$) vardenafil molecules/$\alpha\beta$. This result shows that both the $\alpha$ and $\beta$ catalytic subunits of PDE6 bind vardenafil. In contrast, the same $\alpha\beta$ reconstituted with Py cannot bind $[^3]H$vardenafil to a significant extent under identical experimental conditions (Fig. 3), demonstrating that vardenafil binding is prevented when Py inhibits the catalytic site of PDE6. Preliminary experiments with $[^3]H$sildenafil (35) confirmed the ability of both catalytic subunits to stoichiometrically bind drug, but only in the absence of bound Py (data not shown).

Endogenous Activated Transducin Relieves Py Inhibition of Only One-half of the Full Catalytic Potential of PDE6 on ROS Membranes—To evaluate the extent to which transducin can stimulate PDE6 catalysis and thereby permit binding of $[^3]H$vardenafil to the PDE6 catalytic sites, we used ROS homogenates in which the key proteins of visual excitation (rhodopsin, transducin, and PDE6) remain associated with the disk membrane. The use of ROS homogenates was necessitated by the well established fact that transducin poorly activates rod PDE6 when both proteins are not bound to ROS disk membranes (43, 44). The analysis of these experiments was simplified because PDE6 is the only enzyme present in ROS homogenates capable of breaking down cGMP and of binding vardenafil.

We found that when transducin is inactive (i.e. in the absence of GTP$\gamma$S), PDE6 hydrolytic activity in these ROS homogenate preparations was low (<10% of the fully activated rate) as was the ability of PDE6 to bind $[^3]H$vardenafil (Fig. 4). In addition, limited trypsin proteolysis of ROS homogenates (to fully degrade the Py subunits and thereby activate PDE6 catalysis) resulted in stoichiometric binding of $[^3]H$vardenafil (1.9 ± 0.2 mol vardenafil/mol PDE6 catalytic dimer) and complete activation of cGMP hydrolysis (Fig. 4). These observations agree with the results obtained in Fig. 3 with highly purified PDE6 holoenzyme and catalytic dimer. Surprisingly, transducin activation of PDE6 failed to activate more than 38% + 5% of the full catalytic potential of the enzyme. This level of PDE6 catalytic stimulation by transducin correlated with the observation that only 0.9 ± 0.1 mol $[^3]H$vardenafil/mol PDE6 — 45% of the maximum stoichiometry of 2 mol/mol — was able to access the catalytic sites of transducin-activated PDE6 (Fig. 4). To further explore this relationship between stimulation of PDE6 catalysis by transducin and the availability of the PDE6 active site to bind radiolabeled drug, we added increasing amounts of GTP$\gamma$S to light-exposed ROS homogenates to progressively activate transducin (and hence PDE6). We observed a strong correlation between stimulation of catalysis and an increase in $[^3]H$vardenafil binding as the fraction of activated transducin was increased (data not shown). These results demonstrate by two independent measures (i.e. stimulation of cGMP hydrolysis and accessibility of vardenafil to the active site) that only one-half of the maximum catalytic activity of PDE6 can be disinhibited upon transducin activation.

Because this result differs from the commonly held view that transducin can fully activate PDE6 during visual excitation, we next explored whether the addition of exogenous, activated transducin (i.e. greater than the endogenous levels present in the ROS homogenates) could further stimulate PDE6 catalysis. We purified the $\alpha$-subunit of transducin bound to GTP$\gamma$S ($\alpha$-GTP$\gamma$S) and added increasing concentrations to either ROS homogenates or to purified, soluble PDE6 holoenzyme. As seen in Fig. 5, increasing the concentration of purified $\alpha$-GTP$\gamma$S added to ROS membrane-attached PDE6 (open circles) elevated
PDE6 catalytic activity from 35 ± 4 to 101 ± 1% of the activity of catalytic dimers in which Py had been proteolytically removed. This demonstrates that activated Tα, if present in sufficiently high concentrations (∼1000-fold excess over endogenous levels), can fully relieve inhibition by displacing all Py from its binding sites on the catalytic subunits. Similar results were seen for purified, soluble PDE6 in the absence of ROS disk membranes (Fig. 5, filled circles), except that purified PDE6 holoenzyme is much less efficiently activated by purified Tα-GTPyS (consistent with previous work (43)), as judged by the lower extent of activation of soluble PDE6 compared with membrane-associated PDE6 for any given concentration of Tα-GTPyS.

Conclusions—Two major conclusions emerge from this work. The first is that each catalytic subunit of the PDE6 heterodimer is able to bind ligands to its active site and thus is likely to be catalytically active as well (Fig. 6). This conclusion is based on the innovative approach of utilizing radiolabeled vardenafil to quantify the number of drug-binding sites relative to the cGMP binding stoichiometry for the PDE6 dimer. The conclusion that both the α- and β-subunits are functionally active is further supported by previous work documenting a stoichiometric relationship between Py binding to Pαβ and inhibition of catalysis (i.e. 2 Py bound per Pαβ) (30, 45), as well as cross-linking studies showing Py interacting with both catalytic subunits (46–48). Furthermore, the binding of Py and drug to the active site of the enzyme are mutually exclusive, because high affinity binding of Py prevents radiolabeled vardenafil from binding to the active sites of the PDE6 holoenzyme (Figs. 3 and 4). Although not directly demonstrated in this paper, the above-mentioned data support the inference that both subunits are catalytically active as well. This latter point is further substantiated by the observation that recombinant expression of a monomeric catalytic domain of the structurally homologous PDE5 enzyme (16) is catalytically active (49). Further work is needed to unequivocally demonstrate to what extent each catalytic subunit is catalytically active.

The second major conclusion is that activated transducin efficiently activates only one-half of the potential catalytic activity of the PDE6 catalytic sites and that the remaining activation of catalysis can only be observed when a large excess of activated transducin is present (Figs. 4 and 5). Although our results cannot discriminate the exact mechanism by which transducin activates the PDE6 catalytic heterodimer in this biphasic manner, we present two models that are consistent with our experimental results. The first model (Fig. 6, model B) assumes that activated transducin binds to two independent, nonidentical binding sites on the PDE6 holoenzyme, one of which is efficiently activated by Tα*, whereas the second, low affinity site relieves Py inhibition poorly. This model is consistent with the observation that Py binds to two nonidentical sites on the Pαβ catalytic dimer with ≥10-fold difference in binding affinity (29). The catalytic subunit that binds Py more weakly might correspond to the site where Tα* can more readily displace Py and relieve inhibition at this active site.
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site. The higher affinity site of interaction of PY with the catalytic dimer might require a much higher concentration of Tα* to effectively displace PY to permit catalysis to occur. An alternative model for the biphasic activation of PDE6 by activated transducin is that the binding of Tα* to one catalytic subunit to which PY is bound might allosterically (e.g. negative cooperativity) or stericly reduce the effectiveness with which a second Tα* could bind to and displace PY from the second catalytic subunit (Fig. 6, model C).

The rod photoreceptor outer segment provides a unique cellular milieu for visual transduction and specifically for regulation of PDE6 activation by transducin. The high concentration of transducin (~500 μM) (8) relative to PDE6 (20 μM) ensures the efficiency of propagation of the excitation pathway (3). However, the extremely high affinity of the PY subunits for Pαβ mentioned above (K_D1 < 1 pM and K_D2 = 3 pM) (29) not only prevented spontaneous activation of PDE6 under dark-adapted conditions, it also posed a challenge for transducin to displace PY from its inhibitory site on the PDE6 catalytic domains during light-induced activation of PDE6 catalysis. Our observation that transducin relieves PY inhibition of PDE6 in a biphasic manner (Fig. 5) may underlie physiological differences in the extent of PDE6 activation during transient, dim light stimuli compared with bright, prolonged light stimulation (i.e. light adaptation). For example, at light intensities where only a small percentage of the transducin becomes activated, the ability to efficiently activate PDE6 may be limited to approximately one-half of the full catalytic potential of the enzyme (Fig. 4). This level of activation would still be sufficient to generate a rapid decline in cGMP level, consistent with biochemical and electrophysiological models of the visual excitation and recovery pathways (8). However, prolonged, bright illumination might sufficiently elevate the Tα* concentration high enough to permit the relief of inhibition by PY at its second site of interaction with Pαβ. This idea that biphasic activation of PDE6 by transducin may be correlated with the state of dark versus light adaptation offers another potential biochemical mechanism for the ability of rod photoreceptors to modulate their light sensitivity over several orders of magnitude (50). Although not experimentally addressed in the current study, it is important that future work also examines the possibility that other PDE6-interacting proteins (e.g. glutamic acid-rich protein-2 (GARP2), prenyl binding protein-δ (PDE6), etc.) (2) may modulate transducin-PDE6 interactions as well.

Finally, because mutations in PY have been reported that not only affect its ability to be disinhibited by activated transducin in vitro (51, 52) but also reduce rod photoreceptor flash sensitivity and photoresponse kinetics in vivo (53), the observation of two distinct sites of interaction of transducin with PDE6 holoenzyme has relevance to understanding the molecular basis of loss of visual function and/or retinal degenerative diseases that can result from structural defects in these phototransduction proteins.

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