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Role of Membrane Integrity on G protein-coupled Receptors: Rhodopsin Stability and Function

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Abstract

Rhodopsin is a prototypical G protein-coupled receptor (GPCR) – a member of the superfamily that shares a similar structural architecture consisting of seven-transmembrane helices and propagates various signals across biological membranes. Rhodopsin is embedded in the lipid bilayer of specialized disk membranes in the outer segments of retinal rod photoreceptor cells where it transmits a light-stimulated signal. Photoactivated rhodopsin then activates a visual signaling cascade through its cognate G protein, transducin or Gt, that results in a neuronal response in the brain. Interestingly, the lipid composition of ROS membranes not only differs from that of the photoreceptor plasma membrane but is critical for visual transduction. Specifically, lipids can modulate structural changes in rhodopsin that occur after photoactivation and influence binding of transducin. Thus, altering the lipid organization of ROS membranes may result in visual dysfunction and blindness.

Keywords

G protein-coupled receptor(s); rhodopsin; transducin (Gt); photoreceptor; rod outer segment; membranes; oligomerization; signal transduction; phospholipids; cholesterol; phospholipase A2

1. Introduction

Biological membranes define the boundaries of cells and intracellular organelles and protect them from various harmful substances while providing specific environments for different biological processes essential for life (1). They are composed of lipids, largely phospholipids that form a thin bilayer and embed proteins that are important for the regulation of cellular function and organization in tissues (1, 2). Membranes spontaneously self-assemble with their lipid, hydrophobic tails sequestered inside the bilayer and their hydrophilic heads exposed to the aqueous milieu. The ratio between unsaturated and saturated fatty acids in the bilayer must be well balanced so that the membrane can maintain the fluidity needed for rapid responses to environmental factors. The lipid composition of a membrane affects the structural and functional properties of integral membrane proteins and

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membrane associated proteins as well (2). In this review we emphasize the importance of specific lipids for the function and stability of the light receptor, rhodopsin, a prominent member of G protein-coupled receptor superfamily.

2. Rhodopsin and Gt dependence on membrane integrity

Rhodopsin, a visual photopigment, localizes in disk membranes of rod cells (3, 4) where it is embedded in a lipid bilayer surrounded by ~65-70 phospholipids per one protein molecule (5, 6). After light illumination structural changes in rhodopsin cause coupling to the photoreceptor-specific G protein, Gt or transducin (7), and initiation of the visual signaling cascade (8-10). But does the lipid membrane environment play any role in this activation process? To answer this question, we disrupted ROS membranes by using two detergents, dodecyl- β -D-maltoside (DDM) and n-octyl-glucopyranoside (OG), and then tested the solubilized rhodopsin in a Gt activation assay (Fig. 1A, B). We found that Gt was activated several times more slowly by rhodopsin extracted in DDM than by rhodopsin in native disks (Fig. 1D), whereas no Gt activation was observed when ROS membranes were solubilized with OG (Fig. 1B). However, when lipids isolated from ROS membranes were added back to the ROS membranes in OG, rates of activation were similar to those obtained for native membranes (Fig. 1C). A similar effect of DDM on ROS had previously been reported (11). Interestingly, no changes in the spectral properties of rhodopsin in DDM and OG were observed. Three events, alone or in combination, could account for this result. First, membrane solubilization with detergent may cause dissociation of rhodopsin complexes and thus affect its activation properties. Indeed, our earlier studies suggest that rhodopsin's oligomeric state might be crucial for efficient Gt activation (7, 11, 12). Nonetheless, illumination of only one rhodopsin molecule suffices to produce a neurological response in the brain (13). In this case, the second rhodopsin/photoactivated rhodopsin or opsin would serve as a platform that supports the Gt-rhodopsin complex. Second, a specific lipid environment surrounding rhodopsin may be disorganized due to detergent treatment and formation of mixed micelles composed of lipids, detergent and proteins. Because different detergents sequester varying amounts and populations of lipids, the remaining rhodopsin-associated lipids most likely would affect rhodopsin's functional properties. Third, detergent can affect hydrophobic anchors of Gt (myristoyl and farnesyl groups), and thereby compromise the proper formation of the complex between rhodopsin and Gt. To test the importance of membrane integrity on rhodopsin's properties, we treated ROS membranes with phospholipase A2 (PLA2) that cleaves one acyl chain from the *sn*-2 position of phospholipids. Then we used the digested membranes in the Gt activation assay (Fig. 1E and F). When the efficiency of phospholipid digestion in membranes used for the assay was ~40 %, only slightly slower rates of Gt activation (~16 %) as compared to native ROS membranes were observed (Fig. 1E). However, when we used ROS membranes in which phospholipids were nearly 100 % digested, a far greater decrease (~60 %) in rates of Gt activation occurred (Fig. 1F). Whereas treatment of ROS membranes with phospholipase A2 did not change the spectral properties of rhodopsin, its functional properties were affected. This straightforward experiment clearly indicates that even mild changes in membrane architecture and composition can affect the function of membrane proteins and assigns a significant role to lipids in producing this effect.

3. Rhodopsin oligomerization

Based on classic fluid models for cell membranes and early work on rhodopsin's biophysical properties, this photopigment was viewed for many years as a monomeric protein freely mobile in a liquid-like environment (6, 14-16). But more recent studies on membrane proteins from the GPCR family strongly suggest their propensity for oligomerization (17-23). Although dimerization of family C GPCRs, such as the metabotropic mGlu and

glutamnergic GABA_B receptors, was commonly accepted (24, 25), oligomerization of receptors from rhodopsin-like family A GPCRs has long been debated. Development of high resolution imaging techniques such as atomic force microscopy (AFM) allowed direct visualization of rhodopsin in its native disk membrane environment (26-29). Spectacular AFM images revealed rhodopsin as a dimer organized in rows of densely packed oligomers about 25 nm long embedded in a ROS disk membrane lipid bilayer (Fig. 2). Thus, the simplified view of rhodopsin as a single molecule freely diffusing in a lipid bilayer must be revised in the light of more advanced physiological analyses (30) as opposed to previous measurements (15, 31). Indeed, another powerful technique, near-field scanning optical microscopy or NSOM (32) was used to observe clusters of several β_2 -adrenergic receptor molecules, another family A GPCR, on the surface of neonatal and embryonic cardiac myocytes isolated from mice. This result also strongly suggests that higher organization of rhodopsin-like GPCRs is required for their signaling function as well as their intracellular transport. Moreover, it is consistent with the growing number of *in vivo* investigative reports (e.g. see (21)) and not just those involving experimentally transformed cell lines (20).

Rhodopsin organization can be altered by solubilization of membranes thus, depending on detergent properties, rhodopsin can be found in different oligomeric states in different detergent solutions or the same detergent at different concentrations (5, 12). For example, with the mild non-ionic detergent hexyl- β -D-maltoside (HDM), entire rows of rhodopsin dimers could be isolated as documented by transmission electron microscopy (TEM) whereas another detergent, dodecyl- β -D-maltoside (DDM), disrupted those oligomers into dimers (Fig. 2). Receptor monomers were only occasionally seen. Interestingly, analysis of total phospholipids extracted together with rhodopsin solubilized by HDM and DDM revealed that more phospholipids were sequestered along with rhodopsin in HDM than in DDM. This suggested the importance of phospholipids in stabilizing rhodopsin's quaternary structure. Consequently, addition of lipids to rhodopsin solubilized with DDM promoted rhodopsin's self-association and resulted in its faster migration through a gel filtration column due to oligomer formation (unpublished). After extraction by mild detergents, frog rhodopsin was visualized by blue native BN-gel electrophoresis only as oligomers consisting mostly of dimers and trimers (33), possibly because of its incorporation into detergent-resistant micro-domains. Such micro-domains with distinct lipid compositions have been identified in ROS membranes and most likely play an important role in visual transduction (34).

Implications of the membrane environment for rhodopsin higher organization have been explored by different laboratories. Rhodopsin self-association during reconstitution into lipid vesicles has been tested using fluorescence resonance energy transfer (FRET) (35, 36). Detergent-solubilized, purified and labeled with site-specific fluorophores rhodopsin, self-associated when reconstituted into asolectin liposomes, indicating that lipids play an important role in rhodopsin organization and can promote its self-assembly. Different lipid/protein ratios were tested to ensure that the observed results did not reflect protein crowding. Moreover, light-activated receptor assembly is highly dependent on membrane thickness and is promoted by its reduction. This finding was investigated in more molecular detail by using mesoscopic simulation techniques, where monomeric rhodopsin was incorporated into lipid bilayers composed of phosphatidylcholine (PC) with different acyl chain lengths (ranging from C12 to C20) in different saturation states (37, 38). Interestingly, the number of contact rhodopsin-rhodopsin interfaces was greater in (C12:0)PC and (C16:1)PC than in (C20:0) and (C20:1)PC, suggesting the possibility of non-specific protein-protein interactions in more fluid membranes. However, in thicker bilayers rhodopsin mobility decreased up to 30-fold and only three contact zones were clearly visible in formed aggregates, signifying more specific dimerization interfaces. Dimerization interfaces

between helices H I/H-II/H-8, H-IV/H-V and H-VI/H-VII have been reported for rhodopsin and other GPCRs (19, 39-43).

Therefore, rhodopsin oligomerization is most likely affected by the membrane lipid environment wherein the lipid bilayer acts as a structural matrix that helps to arrange membrane proteins. Reduction of free diffusion in three dimensions to two dimensional biological membranes produces stable complexes even in cases of low affinity interactions. Specifically in ROS membranes, enrichment in phospholipids with long polyunsaturated acyl chains influences this receptor's rigidity and higher order organization. This is not to say that such clustering must be "permanent". Indeed, such islands of receptors can be formed transiently, but they should remain long enough for signal transduction or specifically, interactions with G proteins to take place (44, 45).

4. Lipid composition of ROS membranes

Starting in the 1970s, several laboratories have studied the lipid composition of ROS membranes (46-53) organized as schematically illustrated in Fig. 3A and B. Similar findings revealed that glycerophospholipids are the most abundant constituent comprising more than 85 mol %, or nearly 90 mol % of total lipid in ROS. Moreover, ROS contain ~10 mol % of neutral lipids and ~1-2 mol % of sphingolipids (Table 1).

The most abundant phospholipids are PC and phosphatidylethanolamine (PE) in nearly equal amounts of ~45 mol % and 42 mol %, respectively, whereas phosphatidylserine accounts for ~13 mol %. Other phospholipids such as phosphatidylinositol (PI) and phosphatidic acid (PA) were identified as well but they constitute less than 1 mol % of total lipids in ROS membranes. Interestingly, the distribution of phospholipids in disk membranes is not symmetrical. The inner (luminal) membrane is highly enriched in PC, while the outer (cytoplasmic) membrane leaflet contains more PE and PS (each ~75 % of total ROS PE and PS) (52, 54). Importantly, this asymmetric phospholipid distribution relates to ROS membrane function and stability because it exists in a dynamic equilibrium such that redistribution between two leaflets of ROS membranes may occur during activation of the visual cascade (55). The first enzyme involved in this process is the photoreceptor disk P4-ATPase, Atp8a2, a recently identified phosphatidylserine flippase (56). ROS membranes contain relatively low amounts of neutral lipids, namely cholesterol and neutral glycerides such as diacylglycerol, which account only for ~ 10 % of their total lipids (57). Among these cholesterol is the most abundant species that makes up nearly 90 % of neutral lipids and ~8-10 mol % of total lipids in ROS membranes. Low amounts of sphingolipids have been detected in ROS membranes as well but these represent only 1-2 mol % of total lipids (58, 59). Vertebrate ROS membranes also retain small amounts (2-3 %) of free fatty acids that include 18:0, 18:1, 22:6 (DHA), 16:0, 20:4 and 22:5 (57).

Another striking feature of ROS lipid composition is an unusually high content of long-chain polyunsaturated fatty acids (LC-PUFAs) containing 18-24 carbons and 3-7 double bonds. PE and PS are especially enriched in these PUFAs while PC contains more saturated fatty acids. In mammalian ROS, ~80 mol % of total PUFAs consist of docosahexaenoic acid (DHA) 22:6, which significantly contributes to visual function (60). Moreover, ROS lipids also contain very long-chain unsaturated fatty acids (VLC-PUFAs) with more than 24 carbons (24-36) and 4, 5 and 6 double bonds. VLC-PUFAs account for ~13 % of total PUFAs in ROS and they are mostly found in PC. These fatty acids are very tightly associated with rhodopsin, and thus they might be important for its stability (61). On the other hand, VLC-PUFAs might be involved in the formation of specific micro-domains for cholesterol binding and therefore influence the stability and activity of membrane proteins. Recent studies on fatty acid elongase (ELOVL4) suggest that VLC-PUFAs probably play a

unique role in the retina and may be important for its maintenance and function (62, 63). Specific mutations in the ELOVL4 gene can result in lack of retinal VLC-PUFAs biosynthesis and lead to photoreceptor cell death in Stargardt-like macular dystrophy (64).

5. Lipid-rhodopsin interaction sites

A large number of studies suggest the importance of lipid-rhodopsin interactions for activating visual processes. Lipid composition, especially specific headgroups and acyl chains influence rhodopsin stability, formation of the Meta II active state (see different states of rhodopsin in Fig 3C) and binding of Gt. So what is the nature of the lipid-receptor association? Are there defined regions on rhodopsin's surface that allow binding of specific lipids, which then alter rhodopsin's properties? Can the interaction between rhodopsin and lipids be considered as a receptor-ligand interaction by analogy to other receptors from the GPCR family? In fact, lipid-like ligands have been established for other GPCRs, e.g. 2-arachidonylethanolamide and arachidonylethanolamide are endogenous ligands for the cannabinoid receptor (65). Redistribution of specific lipids within the membrane bilayer after light stimulation has been shown by EPR spin labeling experiments as well (83). Studies of rhodopsin suggest that specific lipid-binding regions exist on its surface. Several well defined phospholipids associated with protein helices near the cytoplasmic ends of H-VI and H-VII have been found in the crystal structure of dark state bovine rhodopsin (66). While the identity of these lipids is unknown because no density was observed for their head groups (possibly dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine), lipid acyl chains did form contacts with specific residues such as Ile²⁵⁶, Ala²⁶⁰, and Ile²⁶³ in H-VI and with Thr²⁹⁷, Tyr³⁰¹, Val³⁰⁴, Ile³⁰⁵, and Met³⁰⁹ in H-VII (Fig. 4A). Helical rearrangements in rhodopsin are followed by activation of this protein, so lipids crystallized together with ground state rhodopsin might be responsible for its stability in the non-active conformation. Moreover, a density into which a cholesterol molecule could fit has been found in 2D crystals of rhodopsin Meta I near the extracellular (luminal) region of H-VI and H-VII of one rhodopsin molecule and the intracellular (cytoplasmic) side of H-IV of the adjacent molecule (Fig. 4B) (67). A specific binding site for cholesterol between transmembrane helices H-II, H-III and, similar to rhodopsin Meta I, the cytoplasmic end of H-IV including a highly conserved Trp4.50 (residue 161 in rhodopsin), have been found in the crystal structure of another family A GPCR, the β_2 adrenergic receptor (β_2 AR-T4) co-crystallized in complex with the inverse agonist, timolol (68). The same area referred to as the cholesterol binding motif in the β_2 adrenergic receptor has also been found in the structure of A_{2A} adenosine receptor; however instead of cholesterol, two stearic acid molecules were bound to this region (69). Moreover, multiple members of family A GPCRs have high sequence similarity among 4 residues participating in the tightest lipid binding. Thus, it seems that a lipid interaction motif is conserved among members of family A GPCRs.

Existence of specific sites on the surface of rhodopsin for interactions with polyunsaturated and saturated lipids, and cholesterol has been reported from 100-ns molecular dynamic simulations (70). These studies indicate that tight coupling between rhodopsin and polyunsaturated DHA occurs frequently in a DHA-rich environment and that this association takes place in small well-defined regions, especially at the extracellular ends of H-VI, H-VII, and H-I (residues 36, 39, 45, 48, 50, 252, 255-256, 259, 266, 269-270, 273-274, 277-278, 286, 290, 300, 304, 307-308, 314). Some residues that tend to associate with DHA have been observed within intracellular ends of H-III and H-IV and the extracellular part of H-II as well (residues 92, 95-96, 99, 129, 130, 133, 136-137, 142-143, 146, 148, 152, 155-156, 159) (Fig. 4Ca,a'). However, cholesterol and saturated fatty acids bound less specifically to rhodopsin. Several groups of residues that preferentially interact with saturated fatty acids have been found within: i) the extracellular ends of H-III and H-IV: residues 108, 111-112, 115, 172; ii) the intracellular end of H-V: residues 217, 220-221,

224-225, 228; iii) extracellular ends of H-V and H-VI: residues 205, 208-209, 213, 273; as well as iv) middle part of H-VI and intracellular end H-VII: residues 256, 300-301, 304-305, 308-309 (Fig. 4Cb,b'). Residues interacting with cholesterol have been found within three clusters: i) H-I and H-8: residues 53, 56-57, 60, 320-321; ii) H-VI and H-VII: residues 252, 255-256, 259-260, 305, 308-309; and iii) HIII and H-IV: residues 108, 111-112, 115, 172 (Fig. 4Cc,c'). Specific interaction sites for cholesterol in the bundle of H-I/H-II/H-VII/H-8 were reported in later studies of rhodopsin done with microsecond molecular dynamic simulations, suggesting a regulatory role of cholesterol in a structural rearrangement needed for the function of all GPCRs (71). Other studies of the interaction between rhodopsin and lipids carried out by saturation transfer difference (STD) spectroscopy in combination with magic angle spinning (MAS) and nuclear magnetic resonance (NMR) (^1H STD MAS NMR) revealed that polyunsaturated and monounsaturated lipids associate specifically with different sites on the rhodopsin surface (72). This technique selectively saturates membrane protein resonance without saturating lipids so magnetization redistributes within the protein via spin diffusion. Magnetization is then transferred from the protein to the first layer of lipids surrounding the protein via ^1H - ^1H dipolar contacts and this requires short distances between protein and lipids equivalent to physical contact between molecules. All rhodopsin photointermediates transfer magnetization preferentially to lipids containing polyunsaturated DHA. Thus, rhodopsin function is influenced by the interaction with specific lipids in the lipid bilayer of disk membranes.

6. Effect of lipids on G-protein binding to rhodopsin

Lipids in disk membranes of ROS not only serve as structural support for rhodopsin but also play a critical role in the activity of this receptor (7). Thus, the lipid composition of the ROS membranes, specifically its asymmetric allocation among disk membrane leaflets, is critical for activating the visual signaling cascade. Both the head groups and acyl chains of phospholipids have a significant effect on Meta II formation (73-79) and G protein anchoring (73, 80-83). Whereas more PC is located in the inner (luminal) disk membrane leaflets, PE and negatively charged PS are enriched in the outer (cytoplasmic) part of the disk. PS is organized in small clusters that help attract G protein to the membrane (84). Even more PS has been found in outer (cytoplasmic) membrane leaflets after rhodopsin photoactivation indicating that phospholipid redistribution may occur in response to light stimuli and most likely stabilizes the active rhodopsin conformation, Meta II (85). Indeed, redistribution of PS was enhanced by the presence of C-terminal Gt peptide that stabilizes the Meta II conformation (86). This indicates a specific role of PS in forming the complex between activated receptor and Gt, most likely by influencing the rhodopsin structure and simultaneously providing a platform for Gt anchoring to the membrane (Fig. 5). Rapid lipid flip-flop depends upon the length and unsaturation of lipid chains, increasing in more fluid membranes rich in long chain polyunsaturated lipids (87). As for fatty acids, ROS membranes are highly enriched in DHA polyunsaturated fatty acid and PS contains more DHA than PE and PC. This seems to agree with the movement propensities of these phospholipids. Very recently an enzyme involved in this flip-flop movement of phospholipids across the membrane was identified: Atp8a2 that belongs to the relatively new subfamily of P-type ATPases. Purified Atp8a2 exhibited ATPase activity stimulated by PS and to some degree by PE but not by PC. When Atp8a2 was reconstituted into liposomes containing fluorescently labeled PS, PE and PC, a flip of PS from the inner (luminal) leaflet to the outer (cytoplasmic) leaflet of the liposome membrane was observed (56).

Movement of PE across disk membrane leaflets following light activation has been suggested as well (85). Using plasmon-waveguide resonance (PWR) spectroscopy, investigators observed that PE favors the formation of Meta II and enhances Gt binding affinity to photoexcited rhodopsin (88-90). In synthetic bilayers composed of a 25:75 mol %

DOPC/DOPE mixture, Gt affinity to activated rhodopsin increased from 64 nM to 0.7 nM, whereas in membranes composed entirely of DOPC, Gt affinity was much lower at 18 nM. Thus, PE most likely affects membrane fluidity, providing more freedom for rhodopsin's structural changes to occur after light illumination, whereas PC inhibits this conversion (88). Free PE serves yet another distinct role in the retinoid cycle by acting as an acceptor for free all-*trans*-retinal released from rhodopsin's chromophore binding pocket after Meta II decay. Formed N-retinal-PE then interacts with ABCA4 (91) and presumably is flipped from the inner (luminal) leaflet to the outer cytoplasmic leaflet of the disk using ATP hydrolysis as the energy source (92, 93). Therefore, PE helps to clear released chromophore from photoreceptor cells for conversion back to 11-*cis*-retinal by the retinoid (visual) cycle (92, 93).

The significance of a specific lipid environment on visual signaling is emphasized by studies of rat ROS membranes (94) when DHA (22:6) was replaced by docosapentaenoic acid (DPA; 22:5 acyl chain). Here, the deficiency of DHA reduced not only rhodopsin activation, but also formation of the rhodopsin-Gt complex in addition to decreasing phosphodiesterase (PDE) activity. Similar observations were reported in rats raised on a n-3 fatty acid-deficient diet (60). Both Gt activation and PDE activity were diminished by lowering DHA levels. Thus, the composition of the lipid matrix is critical, not only for rhodopsin's function, but also for the entire process of signal transduction.

We tested the effect of phospholipids such as asolectin, DOPC, DOPE and DOPS on the binding of Gt to DDM-solubilized rhodopsin following light exposure (11). More efficient formation of the rhodopsin-Gt complex was achieved in the presence of these lipids. DOPS had the greatest effect in this regard, which is not surprising because negatively charged PS derivatives reportedly are far more critical for Gt membrane anchoring than uncharged PC. Phospholipids also appear to be required for rapid Gt activation. Although Gt could be activated by Rho* solubilized in detergent, its activation was several times faster in the presence of either synthetic phospholipids or a mixture of native lipids isolated from ROS membranes. Both DOPS and native lipids resulted in Gt activation rates similar to those observed when Gt was activated by photoactivated rhodopsin in native disk membranes. Thus, these phospholipids most likely play a critical role in stabilizing the quaternary structure of rhodopsin for better membrane anchoring and Gt docking that guarantee rapid signal transduction and immediate responses to light (7, 12, 95).

7. Cholesterol in the function and organization of rhodopsin and Gt activation

Cholesterol is an essential membrane component affecting various functions such as cellular fusion, permeability, enzymatic activity and the operation of various receptors (2). The plasma membrane is highly enriched in cholesterol as compared to ROS membranes in the retina (96). The average cholesterol content in ROS disk membranes is ~8-10 mol % whereas plasma membranes of ROS contain ~30 mol %. Disk membranes in rod cells are formed by evagination of the plasma membrane at the base of outer segments and after separation they move to the apical portion of the ROS within a few days. Old disk membranes at ROS apices are finally shed and phagocytosed by the retinal pigmented epithelium (RPE) (97, 98). The amount of cholesterol substantially decreases as outer disk membranes mature, which has significant implications for activating the visual signaling cascade. Thus, it has been shown that formation of Meta II and binding of Gt to photoactivated rhodopsin is increased in apical disks of rod cells (99), where levels of cholesterol are lower and the amount of phospholipid containing polyunsaturated fatty acids is increased. Rhodopsin accounts for over 90 % of proteins in disk membranes but it is also present in the plasma membrane of ROS, where it constitutes about 40 % of membrane

proteins. Can this plasma membrane rhodopsin activate the visual signaling cascade? A study that compared the activity of PDE resulting from rhodopsin excitation in disk and plasma membranes showed that while disk rhodopsin could fully activate the cascade, activation by plasma membrane rhodopsin was very poor. But when the plasma membrane cholesterol was oxidized to cholestenone, activation of plasma membrane rhodopsin greatly improved (100). This result clearly indicates that a high membrane content of cholesterol inhibits rhodopsin activation. A shift in transition between Meta I and Meta II back toward Meta I was also observed in a reconstituted egg PC bilayer system containing 30 % of cholesterol (101). Because cholesterol increases membrane rigidity, it most likely inhibits expansion of the rhodopsin structure in the membrane during conversion from Meta I to Meta II. Consequently, rhodopsin cannot reach its fully activated state. But cholesterol also helps to stabilize rhodopsin. Therefore, an overall balance of cholesterol must be attained in ROS membranes to ensure rhodopsin's stability without inhibiting its function.

Cholesterol exhibits asymmetric distribution within disk membrane between inner (luminal) and outer (cytoplasmic) bilayer leaflet as well. It can readily exchange between lipid bilayers and is sensitive to phospholipid head groups and fatty acid composition, favoring a saturated fatty acid environment rich in PC but not PE (102). In addition to lipid asymmetry between the bilayers of disk membranes, enrichment of cholesterol in micro-domains called lipid rafts or detergent resistant membranes (DRM) has been reported (103-105). These lipid rafts tend to recruit fatty-acyl modified signaling proteins and probably affect their function. For example, in bovine ROS G α partitions to DRMs as a consequence of rhodopsin photoexcitation, where it can activate other signaling proteins such as PDE. RGS9-1 is a phototransduction regulatory protein that most likely plays a crucial role in terminating G α activity (106, 107). Detailed lipid and protein analyses of DRMs indicate that they are rich in cholesterol, saturated fatty acids, free fatty acids and contain a specific PC fraction devoid of polyunsaturated fatty acids (PUFAs). DRMs hold little PE and PS, and some ceramide-containing PUFAs but no saturated fatty acids. DHA is diminished in PS recruited to DRMs but present in the PE fraction. One of major proteins found in ROS DRMs is caveolin-1. G α and PDE localize to DRMs in a light-dependent manner (108, 109). DRMs isolated by sucrose gradient centrifugation are not rich in rhodopsin but DRM-associated rhodopsin preferentially binds heterotrimeric Gt after light illumination as compared to rhodopsin in bulk lipids. This finding seems to contradict results indicating that enrichment of membranes in cholesterol and saturated fatty acids inhibits the Meta I \rightarrow Meta II transition and formation of the rhodopsin-Gt complex. Nevertheless the observation was confirmed by a second group using fluorescence recovery after photobleaching (FRAP) (34) to study the role of lipid micro-domains in regulating membrane protein function in living rod photoreceptors from transgenic *Xenopus laevis*. Treatment of disk membranes with cholesterol-depleting agents such as methyl- β -cyclodextrin or fillipin III accelerated diffusion of activated GTP-G α as well as heterotrimeric Gt complexed with activated rhodopsin but had no effect on diffusion of either inactive GDP-G $\alpha\beta\gamma$ or ground state rhodopsin. Thus, heterogenic lipid domains must exist in a bulk lipid bilayer and both rhodopsin and Gt are distributed into these domains in a manner dependent on their functional state (106). The complex between rhodopsin and Gt formed as a result of light illumination is either recruited to specific regions or it facilitates lipid reorganization and assembly of micro-domains around itself. This could be the mechanism separating an activated pool of Gt from non-active Gt. Moreover, other studies noted that the inhibitory effect of cholesterol on formation of the rhodopsin-Gt complex is minimized by phospholipids containing DHA which, as mentioned before, is one of the major fatty acids in PS and PE (60, 109). Therefore, a precise ratio between specific lipids surrounding rhodopsin which is balanced according to lighting conditions seems to be crucial for rapid and efficient function of the visual signaling cascade.

8. Role of lipid modification in rhodopsin and Gt structures

Both rhodopsin and Gt possess hydrophobic posttranslational modifications that anchor them to ROS membranes and that are important for their stability and function. Rhodopsin is palmitoylated at two adjacent residues, Cys322 and Cys323, in its C-terminus next to H-8 at the cytoplasmic surface. Interestingly, this palmitoylation is highly conserved among the GPCR superfamily and in rhodopsin this region is involved in binding of Gt following light activation. As shown by studies with *Palm*^{-/-}-deficient mice, absence of palmitoylation causes reduction in rates of Gt activation as well as the forces required to unfold the C-terminal end of rhodopsin, suggesting that stability and/or rigidity of rhodopsin structure was affected (110). Faster phosphorylation rates of depalmitoylated rhodopsin have been observed as well (111). Together, these observations strongly suggest the importance of rhodopsin palmitoylation for proper signal transduction. Moreover, further studies on *Palm*^{-/-}-deficient mice revealed that palmitoylation is critical for stability and correct function of unliganded opsin in rod photoreceptor cells and this hydrophobic modification helps to protect the protein against light induced retinal degeneration (112). The heterotrimeric G protein, Gt contains two different hydrophobic modifications in its structure, a myristoyl and a farnesyl group. The myristoyl group is attached to the N-terminal Gly residue of Gt α and the farnesyl group is attached to the C-terminal Cys71 of Gt γ . Unfortunately both modifications are missing in the crystal structure of Gt but the N-terminus of Gt α and the C-terminus of Gt γ are in close proximity to each other, suggesting that this is a membrane-binding motif. In fact, as determined by electron crystallography, Gt binds to the membrane with a very small contact area (113) that most likely is important for fast signal transduction. Moreover, this result also suggests that most of the Gt's surface is involved in coupling to photoactivated rhodopsin.

In addition to lipid anchors, Gt also exhibits electrostatic interactions with negatively charged membrane surfaces and thus binds more strongly to negatively charged lipids than to neutral membranes (84). Using a quantitative computational approach and calculations of electrostatic free energy, investigators reported that in fact electrostatic repulsion constrains the orientation of membrane-associated Gt. Considering both contacting membranes by lipid anchors and electrostatic interactions with membrane phospholipids, the most preferred orientation with minimum free energy was found to be a "tilted orientation" whereby the N-terminal helix of Gt α is tilted $\sim 30^\circ$ to the membrane surface (114). Different results emanating from future studies may suggest that Gt is able to achieve a number of orientations that ensure an immediate response to light with transduction of the signal.

9. Abnormal lipid composition of the retina and defective vision

An abnormal membrane lipid composition could affect visual signaling. For example, a decreased level of DHA (22:6,n-3) in the retina was found to be associated with a diet low in n-3 fatty acids. Withholding DHA and its precursor, alpha-linolenic (18:3,n-3) acid from the diet produced this effect. Therefore, the lipid composition of biological membranes can reflect the amount and composition of dietary lipids. Rats maintained on diets deprived of n-3 fatty acids were less sensitive to light and evidenced both a significantly reduced capacity for photon absorption by rhodopsin and a greatly decreased rate of rhodopsin regeneration (115). One explanation for this observation could be that rhodopsin's structural organization was disorganized and its function compromised due to altered lipid composition of the lipid bilayer. Reduced rhodopsin activation, inhibited formation of the complex between activated rhodopsin and Gt as well as reduced PDE activation have all been demonstrated in transgenic rats with n-3 fatty acid deficient ROS (94).

Reduced levels of long chain polyunsaturated fatty acids in the blood have been found in humans and animals with inherited retinal dystrophies. Both lipid and fatty acid composition were altered in retina of the Swedish Briard dog that exhibits slowly progressive retinal dystrophy inherited in an autosomal recessive manner. These animals have relatively more PE and PI, but less PC in their retinas than control animals. Analysis of fatty acids in the retina of affected dogs also documented lower levels of n-3 unsaturated fatty acids such as 22:5,n-3 and 22:6,n-3 together with higher levels of n-6 fatty acids such as 18:2,n-6, 20:4,n-6, and 22:5,n-6 (116). There was a direct relationship between the rate of retinal degeneration and the levels of DHA in the ROS. Lower amounts of DHA were found in transgenic rats with either P23H or S334ter rhodopsin – mutations leading to retinal degeneration (117). However, the abundance of major phospholipids in the ROS membranes of these animals was not affected by their disease. Thus, reduction of DHA levels in ROS membranes due to environmental stress or genetic mutations could be considered a neuroprotective mechanism that controls the number of photons captured by rhodopsin and the efficiency of visual transduction where excessive activation can lead to cell death (118).

Perturbations in cholesterol metabolism associated with photoreceptor degeneration have been reported as well (119). In Royal College of Surgeons (RCS) rats, an animal model of retinal degeneration progressing to blindness, it was found that the distribution of cholesterol in rod disk membranes differs significantly from normal animals in which cholesterol was distributed evenly from the base to the apex of ROS. The level of cholesterol in ROS membranes was increased, even though it was decreased in rod cell plasma membranes, most likely resulting from the abnormal phospholipid composition of these rats' disk membranes. PE/PC ratios were 0.6 in the disks and 0.2 in the ROS plasma membranes of RCS rats compared to ratios of 0.9 and 0.4 in normal animals. This result is consistent with the observation that cholesterol partitions out of membranes enriched in PE.

A spectrum of visual defects has been reported for the Smith-Lemli-Optiz (SLO) syndrome in humans characterized by high levels of the cholesterol precursor, 7-dehydrocholesterol, and low levels of cholesterol (120). This defect is caused by an array of mutations with consequences manifested in other tissues besides the eye.

Deficient synthesis of very long polyunsaturated C32-C36 acyl fatty acids (VLC-PUFAs) has been found in autosomal-dominant Stargardt-like macular dystrophy (62). Patients exhibiting this rare disease also carry several mutations in the gene encoding ELOVL4, an enzyme involved in elongation of VLC-PUFAs (59, 121). Therefore, decreased levels of VLC-PUFAs in Stargardt-like syndrome relate to abnormal functioning of ELOVL4 elongase. But the role of VLC-PUFAs is not well understood. Phospholipids containing these fatty acids tightly associate with rhodopsin and possibly stabilize its structure. They also may be involved in clearing retinal compounds from disk membranes after photoexcitation as suggested by results obtained in transgenic, heterozygous ELOVL4 knock-in mice. These mice carrying Stargardt-like associated mutations in their ELOVL4 gene exhibited a deficiency in C32-C36 acyl chains and accumulation of lipofusions in RPE cells following degeneration of rod and cone photoreceptor cells. Surprisingly, homozygous ELOVL4 knockout mice exhibited normal prenatal retinal development but died several hours after birth due to increased skin permeability (64). Thus, VLC-PUFAs may not be required for retinal development but rather for retinal maintenance and function.

10. Conclusions

Rhodopsin remains an ideal model for biophysical and structural studies because it is the only GPCR purified in its native state. But how does a light signal captured by this receptor transfer through the plasma membrane and affect a G protein located 40 Å or more away,

initiating a cellular response? How do changes in the structure and/or dynamics of rhodopsin lead to Gt binding? To answer these questions, a structure that captures this process is needed. Although structures of variously activated individual components of the rhodopsin-Gt complex have been solved at different resolutions, not much is known about the mechanism by which photoactivated rhodopsin binds to its cognate G protein and catalyzes nucleotide exchange. Certainly this system is not limited to just two proteins. Rather additional small molecules play key roles in the functional complex that includes chromophore, nucleotides, lipids, and integral-structural water molecules. As summarized in this review, neutral and phospholipids are predictably critical to this process and they do modulate rhodopsin conformational changes. But they also affect properties of the cognate G protein and are essential for proper formation of the complex between photoactivated rhodopsin and Gt. How we sort out these interactions at a high resolution structural level has remained a technical and intellectual challenge for years. The rhodopsin-Gt system also continues to represent one of the most productive models for investigating membrane biology.

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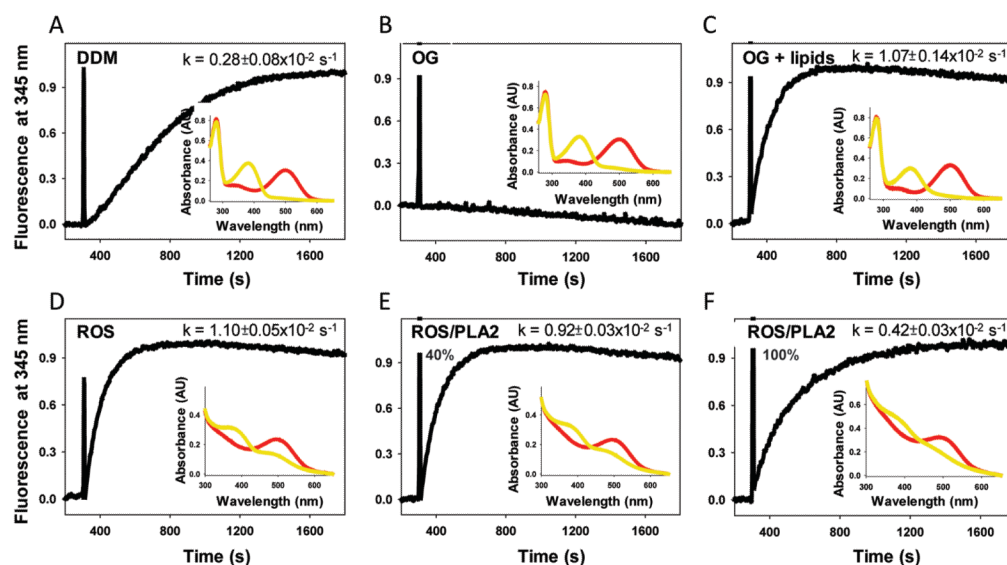


Figure 1.

Ability of different preparations of photoactivated rhodopsin to activate Gt (transducin). Increased intrinsic fluorescence of the Gt α subunit caused by interaction with photoactivated rhodopsin: (A) solubilized in dodecyl- β -D-maltoside (DDM); (B) solubilized in n-octyl-glucopyranoside (OG); (C) solubilized in OG and complemented with lipids isolated from ROS; (D) in ROS membranes; (E) in ROS membranes treated with phospholipase A2 (PLA2) at ~ 40 % digestion efficiency; (F) in ROS membranes treated with phospholipase A2 at ~ 100 % digestion efficiency. Rhodopsin (25 nM) was mixed with Gt (250 nM) and samples were illuminated for 30 s with a fiber light through band pass filter 480-520 nm. After 300 s of recording, 5 μ M GTP γ S was added. Reactions were carried out in a continuously stirred cuvette at 20°C in buffer composed of 20 mM BTP, 120 mM NaCl, 2 mM MgCl₂ plus either 2 mM DDM or 30 mM OG. No detergent was present in the buffer when Gt was activated by ROS membranes. Relative activation rates were calculated from 3 independent experiments. Intrinsic fluorescence of Gt α was measured with a LS55 luminescence spectrophotometer (Perkin Elmer, Life Science), by using excitation and emission wavelengths of 300 nm and 345 nm, respectively (125, 126). *Insets*, Absorption spectra of rhodopsin solubilized in DDM (A), or in OG (B), or in OG and complemented with lipids isolated from ROS (C), rhodopsin in ROS membranes (D) and rhodopsin in ROS membranes treated with phospholipase A2 with ~ 40 % digestion efficiency (E), and rhodopsin in ROS membranes treated with phospholipase A2 with ~ 100 % digestion efficiency (F). Red lines represent spectra of ground state rhodopsin. Yellow lines represent spectra of photoactivated rhodopsin illuminated for 30 s.

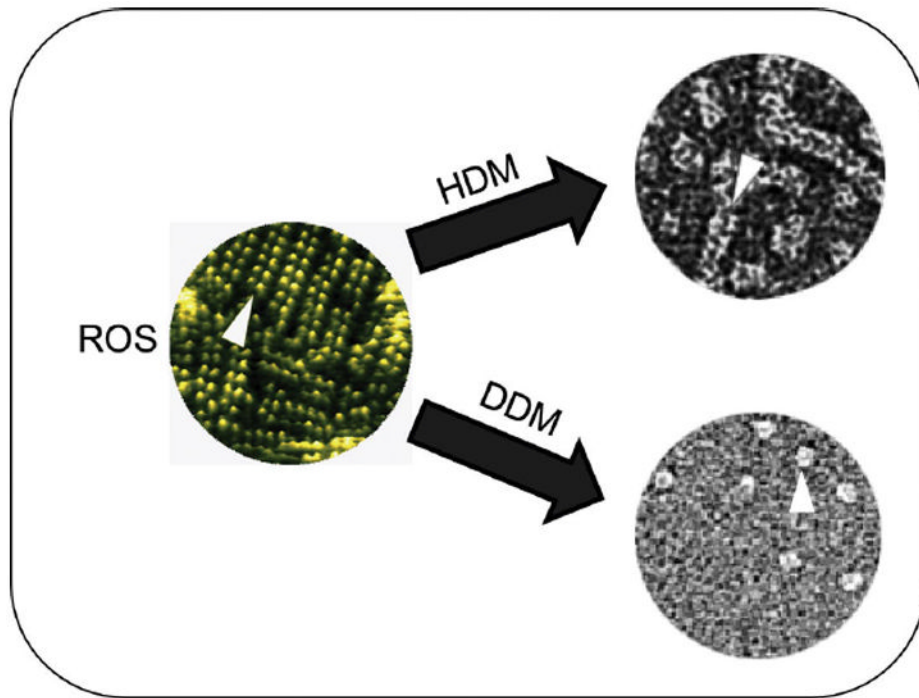


Figure 2. Rhodopsin oligomerization. The propensity of different preparations of rhodopsin to oligomerize is illustrated. Atomic force microscopic (AFM) image of native disk membranes is colored green (left) and organization of rhodopsin molecules in dimers, packed in rows is shown by the arrowhead. Transmission electron microscopic (TEM) images of negatively stained ROS membranes solubilized in either hexyl- β -D-maltoside (HDM) or dodecyl- β -D-maltoside (DDM) are shown on the right. Arrowheads highlight whole rows of rhodopsin dimers extracted by HDM but just single rhodopsin dimers extracted by DDM.

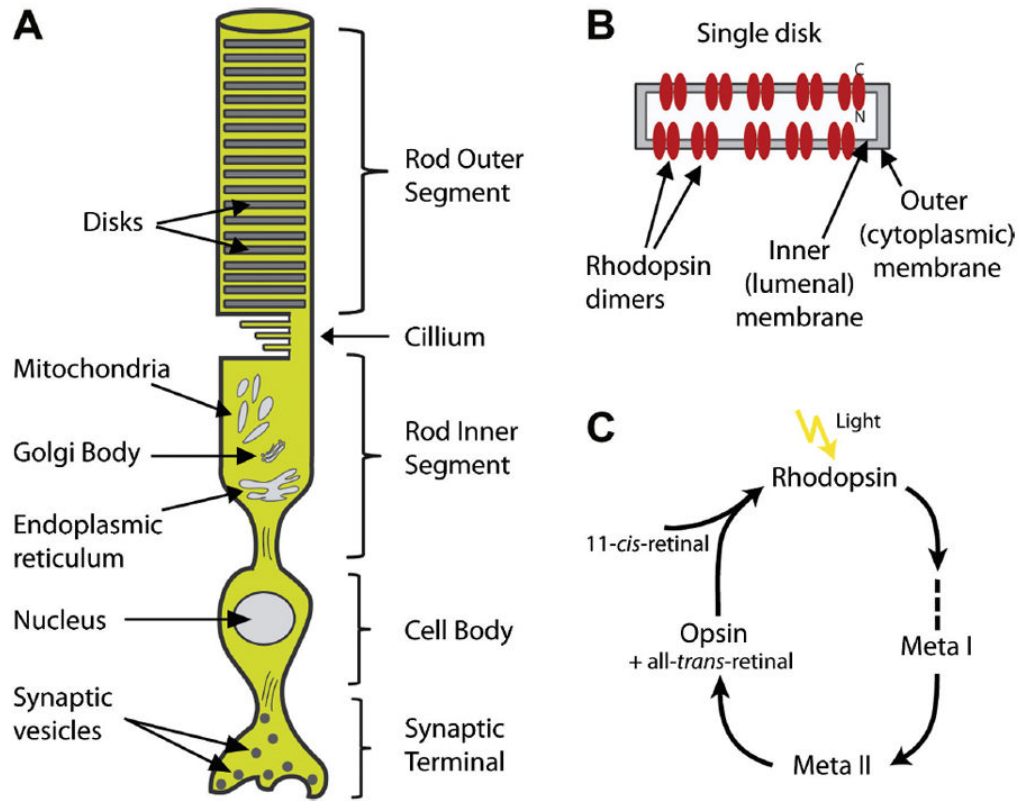


Figure 3. Schematic organization of the retinal rod photoreceptor cell. A, Rod photoreceptor cell with labeled cell segments and major cellular elements. B, Membrane organization of a single disk showing location of rhodopsin dimers. C, Schematic representation of different states of rhodopsin after light activation.

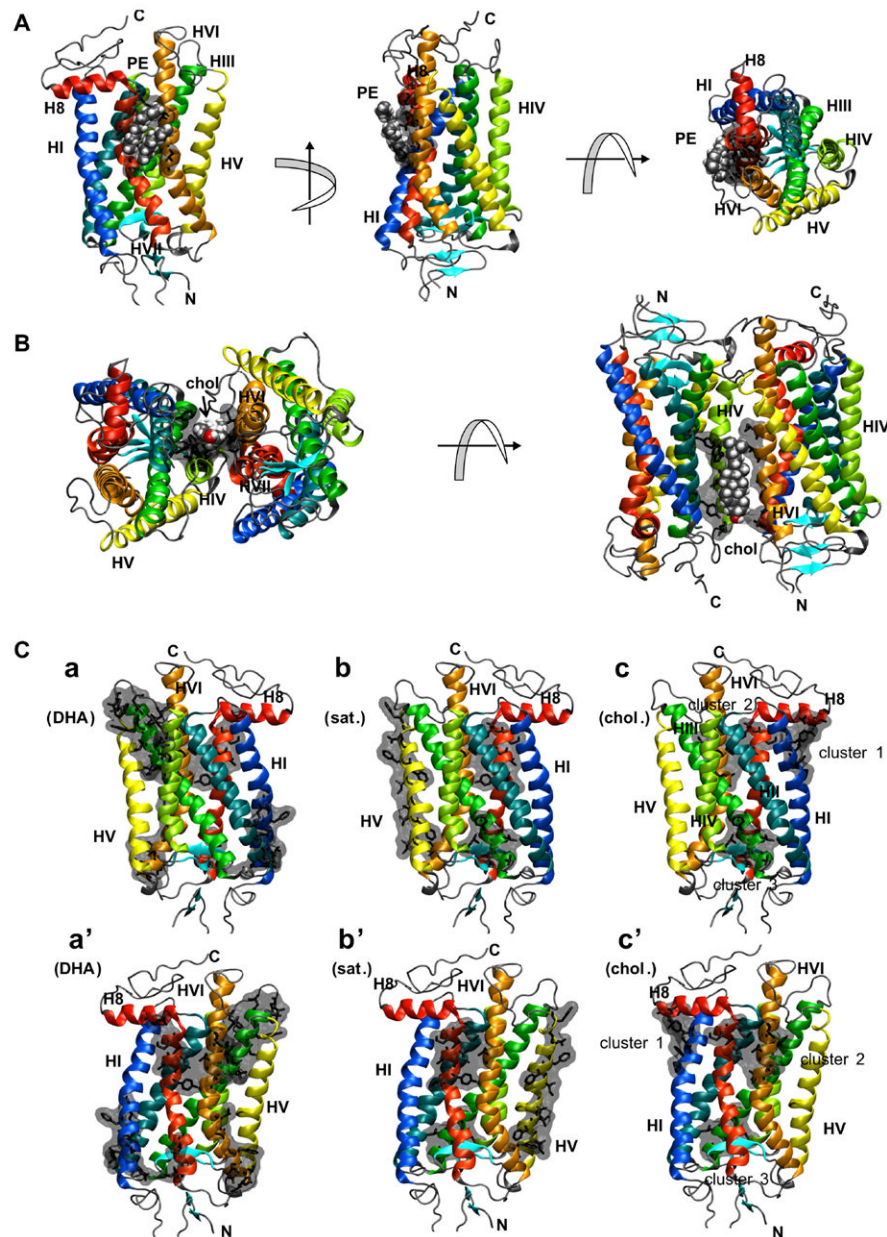


Figure 4. Residues of rhodopsin interacting with specific lipids. (A) Aliphatic lipid chain of dipalmitoyl-*sn*-3-glycero-phosphatidylethanolamine (PE) interacting with cytoplasmic parts of H-VI and H-VII. (B) Cholesterol molecule interacting with two rhodopsin molecules in 2D crystals (antiparallel arrangement of rhodopsin molecules). Cholesterol interacts with the cytoplasmic side of H-IV and the extracellular sides of H-VI and H-VII. (C) Residues of rhodopsin preferentially interacting with DHA (a, a'), saturated fatty acids (b, b'), and cholesterol (c, c'). Primed panels are rotated 180°. The color scheme for helices of rhodopsin is: H-I - blue, H-II - cyan, H-III - green, H-IV - lime, H-V - yellow, H-VI - orange, H-VII - light red, H-8 - red. In A and B panels the lipids are shown with their hydrogen atoms; no lipids in panel C. In all panels the gray shadowing denotes rhodopsin residues interacting with lipids.

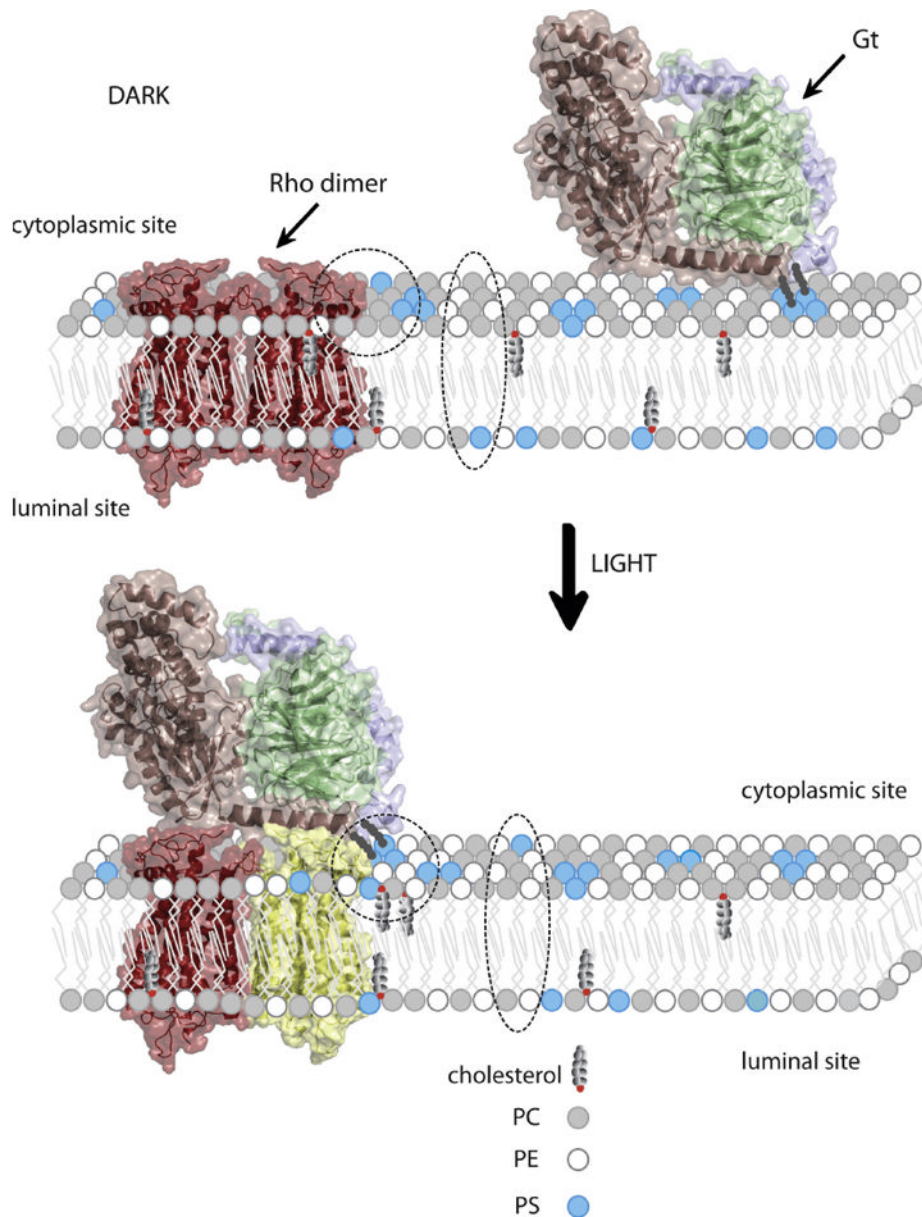


Figure 5.

Reorganization of specific lipids in a disk membrane upon light illumination allows appropriate binding of Gt to photoexcited rhodopsin. Model of rhodopsin dimer (26, 39) is colored red in the dark state. Activated rhodopsin molecule is colored yellow. G α , G β and G γ within heterotrimeric G protein are colored light pink, green and violet, respectively. Gt is attached to the membrane through myristoyl and farnesyl groups. Headgroups of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) are depicted as small circles colored grey, white and blue, respectively. Movement of PS from the inner (luminal) to the outer (cytoplasmic) leaflet of the lipid bilayer, as well as reorganization of PS, PE and cholesterol in close proximity to the activated rhodopsin molecule, is highlighted with a broken ellipsoid and circle, respectively. This lipid reorganization plays an important role in formation of the rhodopsin-Gt complex, thereby activating the visual signaling cascade.

TABLE 1Lipid composition of rod outer segment (ROS) membranes^a.

Phospholipids (~85-90 % of total lipids)		Fatty Acid	
Type	Amount	Type	References
PC	~45 mol %	16:0, 18:0, DHA	(55, 58, 109, 122, 123)
PE	~42 mol %	DHA, 18:0	(55, 58, 109, 123)
PS	~13 mol %	DHA, 18:0	(55, 58, 109, 123)
PI	Less than 1 %	18:0, 24:4, DHA	(55)
PA	Less than 1 %	DHA, 18:0	(55)
Other Lipids			
Cholesterol	~8-10 mol % of total lipids	Not applicable	(55, 109, 124)
Sphingolipids	Less than 1 % of total lipids	18:0, 18:1, 22:4	(55, 109)
Free fatty acids	~2-3 % of total lipids	18:0, 18:1, DHA, 16:0, 20:4, 22:4	(55, 57, 59)

^a **Abbreviations:** PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; DHA, docosahexaenoic acid.