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Differential Rhodopsin Regeneration in Photoreceptor Membranes is Correlated with Variations in Membrane Properties

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Abstract

Rhodopsin, the major transmembrane protein in both the plasma membrane and the disk membranes of photoreceptor rod outer segments (ROS) forms the apo-protein opsin upon the absorption of light. *In vivo* the regeneration of rhodopsin is necessary for subsequent receptor activation and for adaptation, *in vitro* this regeneration can be followed after the addition of 11-*cis* retinal. In this study we investigated the ability of bleached rhodopsin to regenerate in the compositionally different membrane environments found in photoreceptor rod cells. When 11-*cis* retinal was added to bleached ROS plasma membrane preparations, rhodopsin did not regenerate within the same time course or to the same extent as bleached rhodopsin in disk membranes. Over 80% of the rhodopsin in newly formed disks regenerated within 90 minutes while only 40% regenerated in older disks. Since disk membrane cholesterol content increases as disks are displaced from the base to the apical tip of the outer segment, we looked at the affect of membrane cholesterol content on the regeneration process. Enrichment or depletion of disk membrane cholesterol did not alter the % rhodopsin that regenerated. Bulk membrane properties measured with a sterol analog, cholestatrienol and a fatty acid analog, *cis* parinaric acid, showed a more ordered, less “fluid”, lipid environment within plasma membrane relative to the disks. Collectively these results show that the same membrane receptor, rhodopsin, functions differently as monitored by regeneration in the different lipid environments within photoreceptor rod cells. These differences may be due to the bulk properties of the various membranes.

Keywords

Rhodopsin; regeneration; lipid environment; 11-*cis* retinal

INTRODUCTION

Photoreceptor rod cells are responsible for vision under low levels of light. The light sensor, rhodopsin, initiates the visual response as transmembrane protein within compositionally distinct lipid matrices. The fast 11-*cis* to *all-trans* photoisomerization of the chromophore of rhodopsin, retinal, leads to the formation of opsin and *all-trans* retinol. Photoisomerization

is necessary for the formation of Metarhodopsin II [1–3], the activated receptor that couples to the rod cell specific G-protein, transducin [4]. After bleaching, the apo-protein opsin subsequently recombines with 11-*cis* retinal to form a newly activatable rhodopsin. This regeneration of rhodopsin is postulated to be involved in visual adaptation [5] and subsequent activation.

Purified delipidated rhodopsin can only be regenerated in the presence of a detergent [6, 7]. Disk membranes depleted of phospholipid by phospholipase C exhibit a regeneration capacity of only 50%; when phospholipids are added to the system, regenerability is restored [8]. One fatty acid which has a significant affect on rhodopsin regeneration is docosahexanoic acid (DHA, 9). DHA (22:6) or its' precursor (18:3 n –3) must be furnished in the diet [10, 11]. In animal models deprivation of these fatty acids for two generations results in a loss of visual function [12, 13, 14] and ROS disk disruptions [15, 16]. Bush *et al.* [17] have shown that rats with decreased levels of DHA exhibited a slower rate of rhodopsin regeneration when compared to controls. These results suggest that regeneration of rhodopsin is dependent on its' lipid environment.

Rhodopsin is delivered to the ROS plasma membrane from specialized post-Golgi vesicles [18]. It remains associated with the plasma membrane until ROS disk membranes are formed from evaginations of the plasma membrane. Upon disk membrane formation a sorting of the lipid components occurs [19–22]. Cholesterol is enriched fourfold in plasma membrane relative to disk membranes [21] and as the disk membranes age and are apically displaced the cholesterol content of their membranes decreases; from 30 mole% at the base of the ROS to 5% mole at the apical tip [19, 20]. The plasma membrane is composed of predominantly saturated fatty acids, (14:0 and 18:0) with sevenfold less DHA than the disk membranes [21]. Phospholipids are also heterogeneously distributed, with phosphatidylethanolamine accounting for 40% of the total phospholipid in disk membranes and only 11% in plasma membrane [22]. Phosphatidylcholine, is enriched in plasma membranes, accounting for 65% of the total phospholipid, while in disks it accounts for 45% [22]. Thus within the same cell, rhodopsin exists in a variety of membrane environments, with potentially very different dynamic properties.

Although receptors and their associated G-proteins all function in unique lipid environments within different cell types, limited experimental attention has been given to the regulation of receptor proteins in different membranes within the same cell. Evidence of alterations in rhodopsin function due to differences in membrane environment has recently emerged. We have shown that the ability of rhodopsin to activate the cGMP cascade is dependent on the cholesterol content of the disks and plasma membranes [23]. Changes in rhodopsin phosphorylation [24, 25], rhodopsin regeneration [26], recovery from bleaching [27], light responsiveness [28], calcium exchange [29] and cGMP regeneration [30] have been correlated to differences in disk membrane spatial location. In an interesting series of studies, Williams *et al.* [26], have shown species dependent differences in rhodopsin regeneration as a function of disk membrane spatial location. They found that amphibians regenerate rhodopsin faster within basal disks than apical disks. In contrast, rats will regenerate rhodopsin within apical disks faster than in basal disks. While the precise reason for the different trends in regeneration between the species is unknown, it has been

suggested that the differences are due to the availability of retinoids from the villous processes of surrounding epithelial cells [26].

In the present work, we have investigated the ability of opsin to regenerate rhodopsin in bovine ROS membranes. In addition, we have correlated changes in rhodopsin regeneration with differences in membrane dynamic properties, specifically the “fluidity” of the hydrocarbon region and the ordered nature of the cholesterol rich regions in disk and plasma membrane samples. The results presented suggest that rhodopsin regeneration *in vitro* is not dependent on the presence of specific lipids within these membranes but on the dynamic properties of the membranes as determined by total lipid composition.

MATERIALS AND METHODS

Rod Outer Segment Membrane Preparations

For both regeneration and anisotropy experiments, retinal ROS disk membranes were prepared from frozen, dark-adapted retinas (J. Lawson Inc., Lincoln, Nebraska) as described below. Leupeptin (10 μ M) and aprotinin (1 μ M) (both from Sigma, St. Louis, MO) were included in all of the isolation buffers. All manipulations of ROS membranes were performed under dim red light and all buffers were purged with argon to reduce lipid oxidation.

In some experiments ROS disk membranes and plasma membrane were isolated using ricin-agarose and differential sucrose density gradient centrifugation as described [21]. Briefly, purified intact ROS membranes isolated from the 1.11/1.13 g/ml sucrose interface [31] were treated with neuraminidase to expose plasma membrane specific ricin-binding sites. The samples were incubated with ricin-agarose and centrifuged on a 20–50% w/w sucrose gradient with 60% w/w sucrose underlay at 25,000 rpm for 8 hr. The ricin-agarose-bound plasma membrane is sufficiently dense to pellet through the gradient, while the disk membranes banded at approximately 30% w/w sucrose. The ROS plasma membrane was eluted from the ricin-agarose using 1 M galactose in 0.1 M sodium borate, pH 8.0. The plasma membrane (PM) and disk membrane samples were washed in 10 mM Hepes, pH 7.4 (disks at 17,000 rpm, 20 min and PM at 45,000 rpm for 30 min) and resuspended in this same buffer prior to the experiments.

ROS disk membranes were separated based on age and spatial distribution using digitonin as described previously [19, 20]. Briefly, Ficoll floated ROS disk membranes [32] were treated with digitonin in a 1:1 mole ratio relative to the membrane cholesterol. Digitonin differentially perturbs the buoyant density of the disks based on the membrane cholesterol content. Both digitonin treated and control disks were layered onto continuous sucrose density gradients as described [19]. The gradients were centrifuged in a SW-28 swinging bucket rotor at 22,500 rpm for 7hr. The gradients were fractionated and the disk membrane subpopulations were characterized based on their cholesterol to phospholipid ratios.

All of the disk membrane and plasma membrane preparations were resuspended in 10 mM Hepes, pH 7.4 to a final rhodopsin concentration of 1 mg/ml, for both the regeneration and

the anisotropy experiments. Samples used in the anisotropy experiments contained approximately 1–2 μ moles phospholipid/ml.

Measurement of Rhodopsin Regeneration

ROS disk and plasma membrane samples were illuminated with a continuous light source equipped with an orange filter, in the presence of 10 mM Hydroxylamine HCl [33]. Routinely greater than 90% of the total rhodopsin was bleached under these conditions. In all experiments the duration of bleaching was adjusted such that the same total amount of rhodopsin was bleached in the membranes being compared. The bleached membranes were washed twice in 10 mM Hepes, pH 7.4, and the supernatant containing the excess hydroxylamine discarded.

Rhodopsin was regenerated from the apo-protein opsin by the addition of a 2–3-fold molar excess of 11-*cis* retinal (a generous gift from the NEI). The 11-*cis* retinal was added in an ethanol solution, such that the final ethanol concentration was 0.5% (v/v). The samples were incubated in the dark and aliquots were removed at the time points indicated in the figures. The extent of rhodopsin regeneration was measured as an increase in absorbance at 500 nm. The percent rhodopsin regenerated is calculated based on the amount of rhodopsin bleached in each particular experiment.

Labeling of Disk and Plasma Membrane Samples for Fluorescence Spectroscopy

Cholestatrienol (C-3) was incorporated into ROS membranes (disks or plasma membrane) using small unilamellar donor vesicles as described [34]. When labeling disk membranes, the donor vesicles contained 89 mole% phosphatidylcholine (PC, egg, Avanti Polar Lipids), 10 mole% cholesterol and 1 mole% C-3. When labeling plasma membrane samples, the donor vesicles contained 70 mole% PC, 29 mole% cholesterol and 1 mole% C-3 so as not to increase or decrease endogenous levels of cholesterol during the incubation. The samples were washed free of the donor vesicles with three successive washes in 10 mM Hepes, pH 7.4 (17,000 rpm for 20 min). The labeled membrane samples were resuspended in 10 mM Hepes pH 7.4 and the steady state anisotropy determined as described below.

Cis-parinaric acid (*cis*-PA) was added using a minimum volume of ethanol (50 μ l into 3.0 mls of ROS membrane suspensions) at 37°C [35]. Blanks treated with just ethanol were run in parallel with the fluorescently labeled samples. Both *cis*-PA and C-3 were incorporated at probe levels; at a final concentration equal to 0.1–0.3 mole% relative to membrane phospholipid.

Measurement of *cis*-Parinaric Acid (*cis*-PA) and Cholestatrienol (C-3) Steady State Anisotropy

All fluorescence measurements were performed on a Perkin-Elmer LS50 B Spectrofluorimeter equipped with excitation and emission polarizers, at 37°C. Steady state anisotropy (r_{ss}) was calculated [34, 36] using the equation

$$r_{ss} = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}}$$

Where L represents the fluorescence intensity, ν represents the excitation or emission polarizer in the vertical position respectively, h represents the excitation or emission polarizer in the horizontal position respectively. G is the correction factor I_{hv}/I_{hh} . For C-3, G was routinely 1.186 [34]. In the case of *cis*-PA, G was determined independently during each experiment.

Additional Assays

Cholesterol was determined as described [37]. Phosphate was determined as described [38] and modified [39]. Rhodopsin concentration was determined by measuring the difference in absorbance at 500 nm before and after illumination in the presence of 50 mM neutralized hydroxylamine using an extinction coefficient of 40,000 ($M^{-1} \text{ cm}^{-1}$).

RESULTS AND DISCUSSION

Regeneration in ROS Plasma Membrane

We followed the *in vitro* regeneration of rhodopsin following the addition of excess 11-*cis* retinal in plasma membrane and disk membrane samples. The same amount of rhodopsin was bleached (85–90%) in each sample. In the plasma membrane sample less than 60% of the rhodopsin was regenerated after 2 hr (Table I). In the corresponding disk membrane sample, 95% of the disk rhodopsin was regenerated within the same time period. Even after an overnight incubation with 11-*cis* retinal, rhodopsin regeneration in the plasma membrane was never more than 70%.

Regeneration in Disk Membrane Subpopulations

Disk membranes can be separated based on their spatial distribution using sub-solubilizing concentrations of digitonin as described [19, 20]. Using this technique we have isolated disk membranes representing different regions of the ROS. The data in Fig. 1 shows the regeneration of bleached rhodopsin in these different disk subpopulations. The pattern of regeneration is remarkably similar in all of the disk sub-populations. There is a rapid regeneration of 20–40% within the first 5 min of incubation, with the majority of the regeneration occurring within 30 min of incubation. The time-course of this regeneration is consistent with previously published work [33]. While the patterns of regeneration are similar, there are substantial differences in the overall extent of regeneration. Only 45% of the rhodopsin in older disk membranes (cholesterol to phospholipid, mole:mole(c/p) ratio 0.06) was regenerated after a 2 hr incubation with 11-*cis* retinal. Relative to the older disks, disks with a c/p of 0.09 and c/p 0.18 (newly formed) disks showed 80% of the rhodopsin regenerated after the 2 hr incubation. The addition of subsolubilizing concentrations (less than 1 mole%) of digitonin to the disk membranes did not alter the extent of rhodopsin regeneration under these experimental conditions. Control disk membranes; membranes not sub-fractionated based on cholesterol content/age showed greater than 80% regeneration within 60 min. Thus while rhodopsin is present in all disk membranes at essentially the same level, it may differ in its functional properties. Rhodopsin in newly formed disks, those disks which will remain associated with the ROS for at least 6–8 days, is more readily regenerated with 11-*cis* retinal. The rhodopsin in disks at the apical tip of the ROS, soon to be shed and

phagocytosed, is not readily regenerable. These results are consistent with the observations of Williams *et al.* [26] in amphibians.

To determine if rhodopsin regeneration was directly dependent on the cholesterol content of the disk membranes, disk membrane cholesterol was altered using small unilamellar exchange vesicles [40]. We have used this technique to mimic the cholesterol composition of the disk membrane subpopulations [41]. As seen in Fig. 2, increasing the c/p ratio of average disks fourfold to 0.45, did not alter the extent of rhodopsin regeneration. Decreasing cholesterol by half also had no effect on the amount of rhodopsin regenerated. Within 60 min of incubation with 11-*cis* retinal, 85%, 88% and 87% of the rhodopsin was regenerated in control, cholesterol enriched and cholesterol depleted disk membranes respectively. These results suggest that the cholesterol content of the disks does not directly affect the extent of rhodopsin regeneration.

Changes in r_{ss} Reflect Different Membrane Environments Between Disks and Plasma Membrane

The extent of rhodopsin regeneration is dependent on the configuration of the opsin within the bilayer. This configuration is influenced by the lipid environment surrounding the opsin. To determine if differences in membrane properties between disks and plasma membrane may account for the differential regeneration of rhodopsin observed, we determined the “fluidity” of various ROS membrane species using steady state fluorescence anisotropy analysis. As seen in Table II, the fluorescent sterol probe, C-3, reflects a more ordered, less “fluid”, environment in the ROS plasma membrane, than in the disk membranes ($r_{ss} = 0.434 \pm 0.020$ vs. $r_{ss} = 0.152 \pm 0.100$). When disk membranes were artificially depleted or enriched with cholesterol no change in C-3 anisotropy was observed ($r_{ss} = 0.150 \pm 0.002$). We attempted to determine the r_{ss} of C-3 in disk membrane isolated as function of age using digitonin. These experiments could not be done since the C-3 would not exchange into digitonin treated disk membranes. If the C-3 were incorporated prior to the digitonin based separation the r_{ss} results would be uninterpretable due to a digitonin–C-3 interaction. Since C-3 is sensitive to sterol rich regions [34], we also analyzed the fluorescence anisotropy of a fatty acid analog, *cis* PA, to probe the hydrocarbon domains. This probe also showed a significantly higher anisotropy in plasma membrane vs. disk membranes (0.331 ± 0.021 vs. 0.251 ± 0.130) suggesting a more “fluid” environment in the disks.

The precise molecular requirements for regenerability are not known, but the data presented here suggests that the dynamic and structural properties of the membrane rather than specific lipid components are necessary to maintain a regenerable opsin molecule. These results are consistent with earlier studies which showed that the acyl chain length and degree of fatty acid unsaturation are important contributors to rhodopsin regeneration [42, 43], however no single fatty acid species is absolutely required for this process to occur [44]. The data presented exclude a specific requirement for cholesterol in the regeneration process. It is interesting to point out that the same lipid (cholesterol) that affects cGMP hydrolysis through rhodopsin activation [23] does not directly modulate the regeneration (present data) of this protein for subsequent activation.

The anisotropy evidence presented suggests that rhodopsin will regenerate more readily in a less ordered, more “fluid”, environment. These results are consistent with previously published work [6–9, 42–44], if one considers that acyl chain length and degree of unsaturation are important contributors to such bulk membrane properties. In a number of experimental systems cholesterol has the most profound effect on membrane fluidity due to its unique ability to produce a condensing effect (for review see [45]). In the ROS it can be hypothesized that cholesterol induced membrane ordering may be dampened by the high percentage of unsaturated fatty acids, specifically DHA. The plasma membrane contains sevenfold less DHA than disk membranes. Analysis of the results presented here in the context of membrane free volume, suggests that the presence of *cis* double bonds will increase free volume by introducing kinks within the membrane bilayer [46–48]. DHA can contribute 6 such *cis* bonds. As the cholesterol to DHA ratio in the disk membranes decreases as the disk age, a lipid environment is established that favors Metarhodopsin II formation [47, 48] (therefore favors rhodopsin activation of PDEase) and the subsequent regeneration of rhodopsin (high DHA) for continual activation.

We suggest that by utilizing different lipid components to regulate various aspects of rhodopsin function, rhodopsin is maintained in a relatively inactive state in the plasma membrane. In this membrane there is a high level of cholesterol (thus rhodopsin activation is inhibited) and low levels of DHA, (thus rhodopsin regeneration occurs slowly). Upon entering the lower cholesterol, higher DHA environment of the disk through disk biogenesis, inhibition is removed. Thus the plasma membrane lipid environment may serve to “protect” rhodopsin from bleaching by light, thereby allowing for the maximal amount of activatable rhodopsin to be incorporated into newly formed disks.

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Fig. 1. Rhodopsin regeneration in disk membrane subpopulations. The percent rhodopsin regenerated with 11-*cis* retinal in control disks (—●—), and disks with cholesterol to phospholipid mole ratios of 0.18 (—○—), 0.09 (—▲—) and 0.06 (—▽—). The extent of rhodopsin regeneration was measured as an increase in absorbance at 500 nm. The percent rhodopsin regenerated is calculated based upon the amount of rhodopsin bleached in each experiment. The results shown are an average of three individual preparations $\pm$ standard error.

Fig. 2. Rhodopsin regeneration in disk membranes with altered levels of membrane cholesterol. The percent rhodopsin regenerated with 11-*cis* retinal in control disks ($c/p = 0.11$) ($\square$), cholesterol enriched disks ($c/p + 0.32$) ($\blacksquare$) and cholesterol depleted disks ($c/p + 0.05$) ($\hatched$). The percent rhodopsin regenerated is calculated based upon the amount of rhodopsin bleached in each experiment. The results are an average of three individual preparations.

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Table I

Regeneration of rhodopsin in ROS Disk and Plasma Membrane

Sample	c/p* ratio	Rhodopsin Regenerated (%)			
		60 min	120 min	180 min	Overnight
Disk					
Membranes	0.10	70	95	99	100
Plasma					
Membrane	0.32	48	52	65	68

The regeneration of rhodopsin in disk and plasma membrane samples was followed at 37°C following the addition of 11-*cis* retinal, for the times indicated. The extent of rhodopsin regeneration was measured as an increase in absorbance at 500 nm. The percent rhodopsin regenerated is calculated based upon the amount of rhodopsin bleached in each experiment.

^{*} c/p ratio refers to the cholesterol to phospholipid (mole: mole) ratio of the membrane samples.

Table IIThe Steady State Anisotropy of C-3 and *cis*-PA in ROS Disk and Plasma Membranes

	Disk Membrane	Plasma Membrane
r_{ss} <i>cis</i> -PA	0.251 ± 0.013	0.331 ± 0.021
r_{ss} C-3	0.152 ± 0.010	0.434 ± 0.020

The steady state anisotropy of *cis*-PA and C-3 in disk membrane and plasma membrane samples are shown as the mean $\pm$ the standard error of two independent preparations each in triplicate. All anisotropy measurements were carried out at 37°C with an appropriate scatter blank as described in the Methods.