RHODOPSIN LATERAL DIFFUSION AS A FUNCTION OF ROD OUTER SEGMENT DISK MEMBRANE AXIAL POSITION

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ABSTRACT Rhodopsin lateral diffusion was measured at three points along the axis of frog rod outer segments using the method of absorbance recovery after photobleaching. Mean recovery times were slightly longer in distal disk membranes than in proximal disks. A small reduction of pigment mobility with disk age may reflect subtle changes in membrane composition.

INTRODUCTION

Vertebrate retinal rod outer segments (ROS) are continuously renewed by forming disk membranes at the proximal end (1). Adjacent lamellae evaginated from the plasma membrane in the region of the cilium connecting the inner and outer segment seal to form new isolated disks that displace older disks distally (2). Thus the ROS cylinder axis is also a time axis.

Disk structural organization and function are not homogeneous along the ROS axis. Polarized light microscopy demonstrates a birefringence gradient (decreasing in the proximal to distal direction) in the proximal end of amphibian (3–5) and primate (6) ROS. A gradual increase in membrane refractive index has also been found in frog ROS (4), indicating age-correlated changes in disk membrane composition. Studies of toad rod photocurrents using suction micropipettes show position-dependent functional differences as well (7, 8). The sensitivity and speed of local photocurrents are reduced in distal disks when compared with proximal disks. Recovery of photocurrent responses following saturating flash bleaches is also slower in distal disks (9).

The molecular-level basis for any structural, compositional, and functional differences along the ROS axis is unknown. Rhodopsin, the rod visual pigment, is an intrinsic membrane protein that can be used as a probe of the physico-chemical state of disk membranes in several ways. Previous measurements of rhodopsin absorbance spectra and dichroism at different points on the axis of frog ROSs using microspectrophotometry showed no significant differences (4, 10, 11). This means that the concentration, orientation, and chromophore-protein interaction of rhodopsin are invariant within the resolution of the measurement. Rhodopsin has also been used as an intrinsic probe of disk membrane fluidity. Measurements of rotational (12) and lateral (13, 14) diffusion show that rhodopsin is free to spin and move laterally at rapid rates, implying a low viscosity for the disk-membrane lipid matrix. In this study, the lateral mobility of rhodopsin was measured at three positions along the axis of *Rana pipiens berlandieri* ROS, using the method of absorance recovery after photobleaching (15). Results from the three positions were compared to ascertain whether pigment mobility was affected by disk membrane aging. Even though deep disk incisures complicate calculations, this species was chosen because it is the only animal for which the ROS axial gradients of birefringence and membrane refractive index have been described quantitatively (4, 5)

METHODS

Frogs used in this study were maintained at room temperature on a 14-h-light–10-h-dark cycle for at least 6 wk before they were killed. They were force fed twice per week and appeared healthy.

Pigment diffusion was monitored using a kinetic microspectrophotometer. A rectangular aperture was illuminated by a tungsten-halogen source that had been filtered by neutral density filters and a 531 ± 2 nm interference filter to form the probe team. The aperture could also be illuminated by an attenuated 75 W xenon arc source to produce a bleaching white light pulse when a computer-controlled occluder was briefly removed. A second occluder protected the photomultiplier tube during this pulse. After polarization by a Glan-Taylor prism polarizer (normal to the ROS axis), the probe beam was imaged onto the stage plane of a modified Zeiss WL POL microscope (Carl Zeiss, Inc., Thornwood, NY) using an inverted 32x Ultrafluar POL (n.a. = 0.40) objective lens as a condenser. Beam dimensions within the ROS were 2 × 6 μm. The intensity of light collected by a 40x achromat POL (n.a. = 0.85) objective lens was monitored using an EMI 9659QB (S-20) photomultiplier tube (EMI Gencom, Inc., Plainview, NY). Output signals were recorded as a function of time using an EG&G/PAR 4102

waveform recorder (EG&G Princeton Applied Research Corp., Princeton, NJ). Digitized intensity data were monitored on an oscilloscope and transferred to a computer where absorbance values were calculated and routed to a plotter. Cell position relative to the probe beam was monitored using infrared illumination (Kodak Wratten 89B filter, Eastman Kodak Co., Rochester, NY) and an infrared-sensitive television camera and high-resolution monitor.

Cells were positioned so that the probe beam was aligned lateral to the center line in the proximal, middle, or distal regions of dark-adapted, isolated "red" ROS mounted between two coverslips in gelled agar (4). The proximal end of the ROS was easily discriminated either by the presence of an ellipsoid, or by its higher birefringence and distinctive morphology (the cylindrical base is much flatter than the distal tip). Pigment concentration was monitored by recording percent transmission at 100 ms intervals. The dim probe beam produced no perceptible absorbance changes during the period of diffusion measurement (120 s). After establishing a prebleach absorbance baseline, a 0.5 s pulse of white light from the xenon arc lamp was superimposed on the probe beam. The pulse bleached approximately half of the visual pigment exposed to the beam. Rhodopsin lateral diffusion from the unexposed half of the disk membranes was monitored as the recovery of absorbance. Similar to previous studies (13–16), recovery was generally less than half of the initial absorbance loss. This could be due to immobile pigment (16), or to light scattering from the bleaching beam into the opposite half of the disk, or to significant diffusion during the bleaching pulse. The amount of recovery was usually comparable for any series of measurements within the same ROS. Measurements were limited to 100 s postbleach because of distortions caused by the formation of the rhodopsin photoproduct metarhodopsin III. Measurements were repeated in random order for the other axial positions in the same ROS. Temperature was maintained at 23° ± 2°C.

Records of percent transmission as a function of time were converted to absorbance plots $A(t)$. Visual pigment lateral diffusion rates for the proximal, middle, and distal sections were then compared by analyzing plots of the normalized recovery function $R(t) = [A(t) - A(\infty)]/[A(0) - A(\infty)]$, where $A(0)$ is the absorbance at 531 nm immediately following the bleaching pulse, and $A(\infty)$ is the average of fifty absorbance measurements made between $t = 80$ s and $t = 85$ s postbleach, when diffusion equilibrium had been reached. Recovery function time constants $(1/k)$ were computed by a least-squares best fit of single exponential functions (e^{-kt}) to the data. Mean values for k and $1/k$ and their standard deviations were then calculated for each axial point. Cell diameter and disk-membrane invaginations were assumed to be constant along the ROS axis so that intercell and intracell comparisons of diffusion rates could be made.

RESULTS

Diffusion was successfully measured in the proximal, middle, and distal regions of 36 ROS. Plots of the recovery function $R(t)$ decreased in an exponential manner (Fig. 1). Values of recovery half times were in the range of those reported in other studies, so calculated diffusion coefficients ($D \approx 5 \times 10^{-9}$ cm^2/s) would be comparable if the same assumptions are made regarding shape of the diffusing rhodopsin and constraints due to the deep incisures found in *Rana pipiens* disks (13–16).

As seen in Table I the mean recovery time constant $(1/k)$ increased in the proximal to distal direction. While the differences in the mean were less than the standard deviation for the measurement, the difference between the recovery time constants for the proximal and distal points is statistically significant at the $P < 0.001$ confidence level

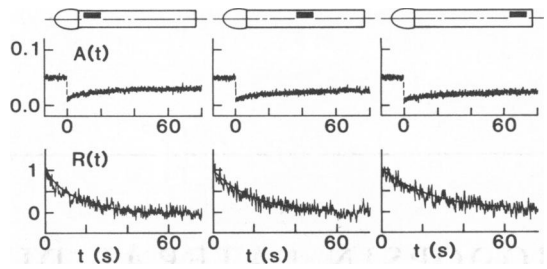

FIGURE 1 *Top* row: Absorbance recovery $A(t)$ at 531 nm after a 0.5-s photobleaching pulse ($t = 0$) measured in the proximal, middle, and distal (*left* to *right*) regions of a "red" rod outer segment. *Bottom* row: Normalized recovery functions $R(t)$ showing the least-squares best fit single exponential curves (e^{-kt}) used to compare lateral diffusion rates at the three points.

when analyzed using the Student's t test (17). Also the three recovery time constants measured for each cell were in ascending order in the proximal-to-distal direction for 12 of the 36 cells. There is less than one chance in 100 that this would be the case for the assumed conditions of random variation from a uniform diffusion rate. The histograms in Fig. 2 compare the distributions of $R(t)$ time constants for the proximal and distal points. While there was considerable overlap, the histogram for the proximal points was centered around lower values than the histogram for the distal points. Therefore it is very likely that rhodopsin lateral diffusion in *R. pipiens* is slightly slower, on the average, in the older distal disk membranes than in the newer proximal disks.

DISCUSSION

Brief reports from other laboratories state that no differences were found for rhodopsin lateral diffusion rates as a function of axial position in *Rana catesbiana* (16) or *Bufo marinus* (18) ROSs. The small difference reported here for *R. pipiens* ROSs may be species specific, but it is more likely that the data treatment and analysis used in this study were responsible for discriminating a statistically

TABLE I
MEAN RECOVERY FUNCTION TIME CONSTANTS $(1/k)$ AT THREE POSITIONS ON THE ROS AXIS

	Proximal	Middle	Distal
	s	*s*	*s*
Mean recovery time constants	15.7	16.4	20.0
Standard deviation	4.7	5.5	4.9
Standard error	0.8	0.9	0.8
		*	‡
		§	

*Differences are not significant at the $P < 0.01$ level using Student's t test.
‡Difference is significant at the $P < 0.01$ level.
§Difference is significant at the $P < 0.001$ level.

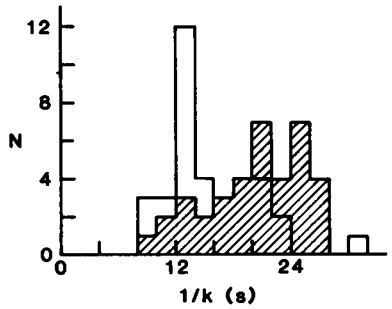

FIGURE 2 Histograms of the recovery function time constants ($1/k$) measured in the proximal and distal regions of 36 ROS. While there is considerable overlap, the distal region histogram is significantly shifted to longer recovery times (slower diffusion rates).

significant trend toward disk age-correlated mobility changes.

It is reasonable to speculate that age-correlated functional changes necessitate the continuous production of new disks. Rod cells do so at a furious pace and at great metabolic cost, suggesting how important maintaining the properties of young disk membranes is to visual function. Disk membrane phospholipids have exceptionally high levels of polyunsaturated fatty acids that presumably impart the low viscosity found in such membranes (19). These fatty acids are especially susceptible to peroxidation because of the high oxygen tension and light levels found in the retina. Partial protection from peroxidation is probably afforded by high alpha-tocopherol (vitamin E) levels (20), but it is reasonable to expect some degradation that could affect the molecular environment of rhodopsin and other proteins involved in visual transduction. It seems unlikely that the small reduction in mobility found here can be directly responsible for any significant age-correlated functional change. However, the data do reflect the fact that subtle changes in the disk membrane matrix are occurring that may degrade the transduction or recovery mechanisms.

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