LATERAL DIFFUSION OF RHODOPSIN IN PHOTORECEPTOR CELLS MEASURED BY FLUORESCENCE PHOTOBLEACHING AND RECOVERY

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ABSTRACT Frog rod outer segments were labeled with the sulfhydryl-reactive label iodoacetamido tetramethylrhodamine. The bulk of the label reacted with the major disk membrane protein, rhodopsin. Fluorescence photobleaching and recovery (FPR) experiments on labeled rods showed that the labeled proteins diffused rapidly in the disk membranes. In these FPR experiments we observed both the recovery of fluorescence in the bleached spot and the loss of fluorescence from nearby, unbleached regions of the photoreceptor. These and previous experiments show that the redistribution of the fluorescent labeled proteins after bleaching was due to diffusion. The diffusion constant, D, was $(3.0 \pm 1.2) \times 10^{-9}$ cm^2 s^{-1} if estimated from the rate of recovery of fluorescence in the bleached spot, and $(5.3 \pm 2.4) \times 10^{-9}$ cm^2 s^{-1} if estimated from the rate of depletion of fluorescence from nearby regions. The temperature coefficient, Q_{10}, for diffusion was 1.7 ± 0.5 over the range 10°–29°C. These values obtained by FPR are in good agreement with those previously obtained by photobleaching rhodopsin in fresh, unlabeled rods. This agreement indicates that the labeling and bleaching procedures required by the FPR method did not significantly alter the diffusion rate of rhodopsin. Moreover, the magnitude of the diffusion constant for rhodopsin is that to be expected for an object of its diameter diffusing in a bilayer with the viscosity of the disk membrane. In contrast to the case of rhodopsin, FPR methods applied to other membrane proteins have yielded much smaller diffusion constants. The present results help indicate that these smaller diffusion constants are not artifacts of the method but may instead be due to interactions the diffusing proteins have with other components of the membrane in addition to the viscous drag imposed by the lipid bilayer.

INTRODUCTION

Cell membranes may be viewed as fluids whose constituent molecules move freely in two dimensions. This lateral mobility is probably important in the function of cell membranes, for example in the transmission of hormone signals from the exterior to the interior of the cell (1), and in coupling components of enzyme-acceptor systems in the endoplasmic reticulum (2) and cell surface (3). Reaction rates in some or all of these systems may be diffusion limited and hence it is of interest to measure lateral diffusion coefficients of membrane constituents, especially membrane proteins.

The first diffusion coefficient determined for a membrane protein, vertebrate rhodopsin, was found to be $D = 3.5 \times 10^{-9}$ cm^2 s^{-1} (4), a value consistent with membrane lipid viscosity. In contrast most fluorescence photobleaching and recovery (FPR) measurements of the rate of lateral diffusion of membrane proteins often yield coefficients at least tenfold smaller than expected from membrane lipid viscosity, raising the possibility that the FPR method may

artifactually slow the diffusion of the labeled proteins (5, 6). The lateral diffusion of rhodopsin was observed by measuring the time required for native rhodopsin to return to a portion of rod outer segment (ROS) disk membranes in which the rhodopsin had been bleached by a brief exposure to bright light. Both the depletion of native rhodopsin from unbleached portions of disks and its reappearance in the bleached area could be observed photometrically. Since rhodopsin contains a native, bleachable chromophore, these measurements could be done on fresh, unmodified ROS. However, this method can not be used for membrane proteins in general. The FPR method was developed to study membrane proteins in general by following the approach described above but using exogenous fluorophores instead of endogenous chromophores as markers for the membrane proteins of interest. In these experiments, membrane proteins covalently labeled with fluorophore are briefly bleached by a laser beam in a small area of the membrane and the diffusion rate is determined from the half-time of recovery of fluorescence in the bleached spot (7) measured with the beam attenuated so as to bleach only a negligible amount of the remaining fluorophores.

As noted above, the diffusion coefficients obtained in such FPR measurements are in many cases an order of magnitude or more smaller than that observed by bleaching native rhodopsin (reviewed in references 5 and 6). There are indications that lateral diffusion of most membrane proteins is restricted not only by the viscosity of the lipid bilayer but also by other elements associated with the cell membrane (8, 9, 17). However, it is important to learn if the FPR method introduces artifacts, for example due to membrane damage by the bleaching laser light, or to photochemical cross-linking, which might result in seriously underestimating D. One control for such an artifact is to compare D obtained for a particular protein in cells that have been previously bleached with D obtained for the same protein in cells bleached for the first time during the measurement (10).

Here we present the results of a second control experiment which indicates that the FPR method itself does not yield artifactually low diffusion coefficients: our results using the FPR method on rhodopsin diffusing in ROS disk membranes are in good agreement with the results obtained earlier using the photometric technique on fresh intact ROS.

MATERIALS AND METHODS

The fluorescent labeling reagent, iodoacetamido tetramethyl rhodamine, was obtained from Research Organics Inc. (Cleveland, Ohio), and used without purification. Rhodamine 110 ($C_{20}H_{15}ClN_2O_3$) was obtained from Eastman Kodak Co. (Rochester, N.Y.). Retinae were dissected from bullfrogs (*Rana catesbiana*). ROS were isolated by gently shaking a retina in a small volume of frog Ringer's solution (3 mM TES, 114 mM NaCl, 2.5 mM KCl, 5 mM dextrose, pH 7.4). Then 2 ml of freshly prepared fluorescent labeling solution (~25 μM in Ringer's solution, pH 7.4) was added to the ROS suspension in the dark. The molar ratio of label to rhodopsin was ~10. The reaction mixture was incubated for 20 min at room temperature. Then the labeled ROS were washed four times with a small volume (~2 ml) of Ringer's solution, and transferred to rectangular glass capillary tubes with a path length of 0.05 mm (Vitro Dynamics, Inc., Rockaway, N.J.). At this level of labeling (concentration and time), the ROS were not visibly damaged, as seen through a microscope, even after several bleaches, each with a flash duration of 20 ms, 70% bleach. When ROS at comparable concentrations were labeled with either tetramethyl rhodamine isothiocyanate, (Research Organics Inc.), or rhodamine 110, the ROS were usually damaged by a few bleaches when examined with the microscope. Using the same light exposures, unlabeled ROS were much more resistant to bleaching damage.

Fluorescence was measured on a Leitz (Ortholux) microscope (E. Leitz, Inc., Rockleigh, N.J.). The

514-nm beam of an Argon-Krypton Laser (Model 552, Control Laser Corp., Orlando, Fla.) was used to excite fluorescence, from above the specimen, via a ×40 objective with a numerical aperture of 1.3. The laser beam was focused to a spot 2.2 μm in diameter ($1/e^2$ width) on the specimen. The fluorescence was collected through the objective and a dichroic mirror and measured by a photomultiplier (EMI GenCom, Inc., Plainview, N.Y.) operating with a photoncounter (PAR Instruments Model 1140, Princeton, N.J.). The counter output was recorded on a fast pen recorder (Hewlett-Packard, Palo Alto, Calif.). The effects of temperature on diffusion were followed using a thermoelectrically cooled and heated microscope stage (Bailey Instruments Model TS2, Saddle Brook, N.J.).

To calculate the lateral diffusion coefficient, D, we used two procedures. First, we followed a modification of the procedure described by Axelrod et al. (7) (Fig. 1 *a*). Since the optical axis of the beam is perpendicular to the long axis of the ROS, i.e., parallel to the disk membranes, the diffusion of rhodopsin molecules into the bleached area can be treated as being one-dimensional, instead of two-dimensional. Therefore, Eq. 19 of Axelrod et al. (7) becomes:

$$D = \frac{\omega^2}{2t_{1/2}} \gamma_D, \tag{1}$$

where ω is the half-width at e^{-2} height of the Gaussian laser beam (1.1 μm), $t_{1/2}$ is the half-time of the FPR, and γ_D is a numerical factor depending on the amount of bleaching. In this case, $\gamma_D \simeq 1.3$ (70% bleaching).

In the derivation of Eq. 1, the boundary condition used is that for an infinite system, i.e., a cell much larger than the beam width. This is a poor approximation for a ROS, since its radius is only 4 μm.

Therefore we chose to solve the diffusion equation by numerical analysis for a finite system (11). We find that for a Gaussian beam (ω = 1.1 μm) and disk membrane radius R of 4 μm:

$$D = \frac{f R^2}{t_{1/2}\pi^2}, \tag{2}$$

where f is a weak function of ω and R; in this case $f = 0.3$. (In addition, we found that for a beam size of up to 40% of the cell size, the ratio between the Ds calculated from Eqs. 1 and 2 is a linear function of beam size, i.e., D (Eq. 2)/D (Eq. 1) $\simeq 1 - 1.8\ \omega/R$).

To apply Eq. 2 to disk membranes, we have to consider some additional factors: (*a*) the irregular shape and boundary of the disk membrane, and the numerous fissures in disk membranes which can slow lateral diffusion. To correct for these, we have performed experiments in which the diffusion of particles in the disk was modeled by the diffusion of heat in an aluminium disk cut to the form of a typical ROS disk (14). These model experiments showed that a correction factor of 1.9 was needed in Eq. 2 to account for the slowing effects of both the disk shape and the fissures. (*b*) The laser beam does not propagate through the ROS as a parallel beam, for which Eq. 2 was derived. Rather it converges and diverges, forming a minimum "waist" near the center of the disk membrane. The actual profile of the beam was measured by viewing a thin film of fluorophores. The width of the fluorescent spot was measured as the position of the film was varied near the focal plane and the resulting cross-section of the beam profile is shown in Fig. 1. The thin-film was made by placing a drop of methanol solution of rhodamine on a microscope slide and allowing the methanol to evaporate. The effect of this measured beam profile on the calculated value of D was estimated by numerical methods to reduce f in Eq. 2 by a factor of 0.83. This correction accounts both for the circular (Gaussian) cross-section of the beam and the increase in its width above and below the focal plane. In calculating this correction factor, the fluorescent label was assumed to diffuse only in the plane of the disk membranes (4).

In the second procedure for estimating D we observed the loss of fluorescence from a spot a few microns to the side of the bleached region (Fig. 1 *b* and *c*) instead of fluorescence recovery in the bleached region. The measuring beam was shifted a few microns by inserting an optical wedge (constructed from two microscope slides and a spacer, and filled with mineral oil). D was calculated from the half-time ($t_{1/2}$) of the loss of fluorescence from this unbleached region using a previously

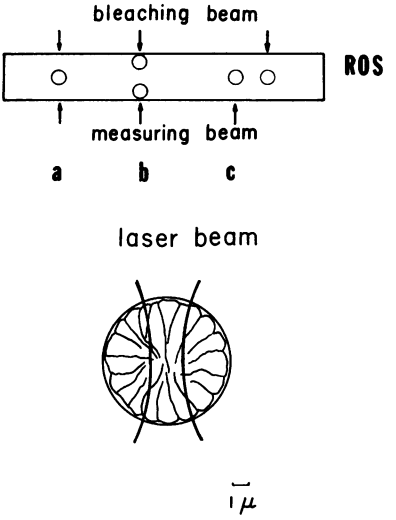

FIGURE 1 (top) Diagram of the arrangements of the measuring beam and the bleaching beam in the FPR experiments. (*a*) Conventional type, where measuring beam and bleaching beam are coaxial. (*b* and *c*) Measuring beam is to the side of bleaching beam, across or along the axis of the ROS. (bottom) Diagram of the cross-section of an ROS with the fissures, and the laser profile for the arrangement *a*. Bar, 1 μm.

derived formula. According to Poo and Cone (4):

$$D = \frac{0.69\,(2R)^2 b}{\pi^2 t_{1/2}}, \tag{3}$$

where R is the radius of the ROS, and b is the geometric factor that accounts for the underestimation of the D in disk membranes because of the fissures ($b = 2.7 \pm 1$ for frog).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis of labeled ROS was carried out as previously described (12, 13). Fluorescent gels were photographed through a red barrier filter.

RESULTS

ROS labeled with iodoacetamido tetramethylrhodamine appear uniformly fluorescent, with an intensity about equal to that measured from a 50 μM dye solution in ethanol in a 50-μm pathlength cuvette. No ring or outline stain was seen, indicating that the disk membranes were labeled as well as the surrounding plasma membrane. SDS gel electrophoresis showed that the bulk of the label was associated with rhodopsin, though some free dye was detectable as well as label on other proteins (Fig. 2). Pretreatment of ROS with the sulfhydral reagent iodoacetamide or *p*-chloromercuribenzoate reduced, but did not completely block, labeling.

Fig. 3 shows a typical FPR experiment, showing recovery of fluorescence after bleaching. On the average, fluorescence recovered to ~40 ± 12% of the initial intensity with a half-time of 3.1 ± 1.0 s at 22°C. The half-time of recovery increased 1.7 ± 0.5-fold for every 10°C decrease in temperature over the range 10°–20°C. Fixation with glutaraldehyde (8% for 10 min) blocked recovery of fluorescence (Fig. 4); formaldehyde (5%, 10 min) fixation had no detectable effect on recovery. The recovery of free rhodamine 110 was almost complete with a half-time of ~0.6 s (Fig. 4); this recovery was not blocked by glutaraldehyde fixation.

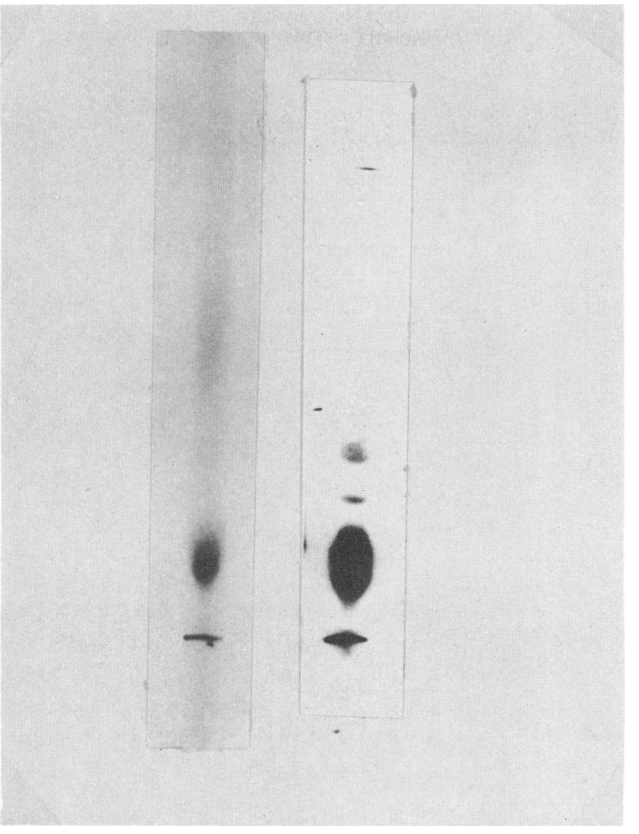

FIGURE 2 SDS gel of fluorescent labeled proteins solubilized from ROS. (right) Fluorescence pattern. (left) Coomassie blue stained pattern.

In addition to monitoring the return of fluorescence to the bleached spot, we followed depletion of fluorescence from a nearby spot never exposed to the bleaching light. When the spot monitored was displaced transverse to the long axis of the ROS (Fig. 4), fluorescence from the spot diminished with a half-time $t_{1/2}$ of 23 ± 5 s at 22°C (Fig. 4, top). No change in fluorescence intensity was detected when the spot was displaced parallel to the long axis of the ROS (Fig. 4) as expected since rhodopsin has previously been shown to be confined to each disk and is unable to diffuse along the ROS axis.

Using Eq. 2 and including the correction factors mentioned above, we estimate $D = (3.0 \pm 1.2) \times 10^{-9}$ cm^2 s^{-1} (>100 measurements). Using Eq. 3 we estimate $D = (5.3 \pm 2.4) \times 10^{-9}$

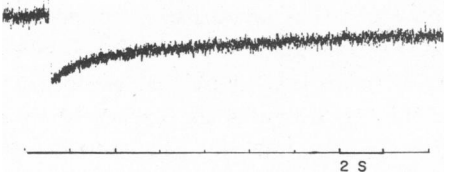

FIGURE 3 A typical record of FPR. Fluorescence intensity at 590 nm as a function of time. Bar, 2.0 s.

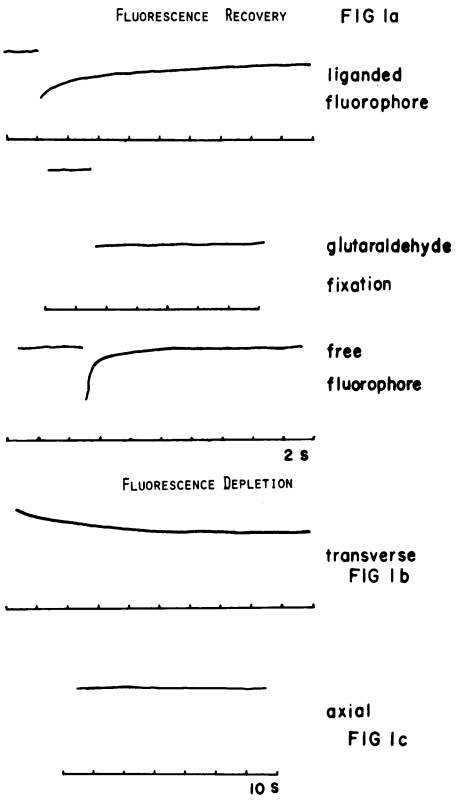

FIGURE 4 Tracings of original experimental records of FPR-fluorescence intensity at 590 nm as functions of time. The fluorescence recovery experiments were performed with arrangement *a* of Fig. 1. (top) ROS labeled with iodoacetamido tetramethyl rhodamine. (middle) ROS labeled with iodoacetamido tetramethyl rhodamine and then fixed with glutaraldehyde. (bottom) ROS labeled with free dye, rhodamine 110. The time scale is 2.0 s. The fluorescence depletion experiments were performed with arrangements *b* and *c* in Fig. 1. The time scale is 10.0 s.

$cm^2\ s^{-1}$ from loss of fluorescence from a nearby spot (nine measurements). The uncertainties in these calculated values are estimated from the combined uncertainties in measuring $t_{1/2}$, ω, and R and the uncertainties in the correction factors for disk fissures and beam profiles.

DISCUSSION

Reaction of ROS with the sulfhydryl label iodoacetamido tetramethylrhodamine primarily labels rhodopsin in disk membranes of the ROS. Though some dye remains unbound to protein, either in membrane lipids or in solution between disks, our measurements of fluorescence recovery after photobleaching appear to detect primarily the diffusion of labeled protein. Half-times for recovery of fluorescence in ROS labeled with free, unreactive rhodamine 110 were five times faster than those that we measured with covalently bound fluorophores and recovery of the free label was not blocked by glutaraldehyde fixation of the labeled ROS. Since the electrophoretic analysis also indicates that the bulk of the ROS membrane is rhodopsin and that the greatest amount of label is associated with rhodopsin, the FPR measurements described here report primarily the diffusion of rhodopsin.

The following evidence indicates the recovery of fluorescence in the bleached area was due to lateral diffusion, and not to photochemical recovery of the bleached label or to metabolic activity of the ROS: (*a*) The fluorescence not only recovered in the bleached spot, but also diminished in unbleached areas of the same disks (Fig. 4). Moreover the fluorescence from disks above or below the bleached spot did not change (Fig. 4). (*b*) The recovery of fluorescence was blocked by fixation of the ROS with a cross-linking fixative glutaraldehyde; such fixation is unlikely to affect the chemical recovery of fluorescence. (*c*) Formaldehyde fixation adequate to disrupt metabolism did not slow the recovery of fluorescence.

The diffusion coefficients we obtain from both fluorescence recovery and from fluorescence depletion are in good agreement (3.0 ± 1.2 vs. $5.3 \pm 2.4 \times 10^{-9}$ cm^2/s). It is worth noting that the value for D obtained with the fluorescence depletion measurement is principally a function of the geometry of the disk surface which contains the diffusing molecules and is relatively insensitive to the width of the bleaching beam whereas the value for D based on recovery of fluorescence is more sensitive to the size of the bleaching beam relative to the size of the disk membranes (2.2-μm diam beam on a 8-μm diam disk). However, the good agreement between the two values for D indicates that there is no serious error introduced by the beam size.

On average, fluorescence in the bleached spot recovered to $40 \pm 12\%$ of the initial intensity. This level is similar to the fractional recovery found for other membrane proteins in larger cells (5, 6). However, in the small ROS a significant fraction of label is destroyed by the bleaching light and we calculate that only 52–61% recovery would be seen if all the labeled rhodopsin molecules were free to diffuse (for a 2.2–2.7-μm diam beam and an 8-μm diam cell). Thus our results indicate that at least ~66–77% of the rhodopsin was mobile in the plane of the disk membrane. If the bleaching beam size has been underestimated, or if the fissures in the disks affect total recovery this may account for the remaining "immobile" fraction. However, we suspect that other factors influence the observed fractional recovery of fluorescence. The degree of recovery of fluorescence after bleaching appeared to depend upon the condition of the animal used, the handling of the sample, and the position along the ROS axis that was measured. The fractional recovery of fluorescence was usually largest near the base of an ROS and decreased towards the other end. This observation may be related to the findings that new disk membranes are continuously being synthesized at the base of the ROS (15), and that the birefringence of a ROS varies along its length (16).

In conclusion we have determined the lateral diffusion coefficient of rhodopsin in the disk membrane by the FPR method, and we observe a value which is in good agreement with the value obtained from fresh, unlabeled ROS with the rhodopsin bleaching method. Thus the FPR method did not significantly alter the rate of diffusion of rhodopsin in disk membranes. This suggests that the relatively slow diffusion of membrane proteins often observed by the FPR method is less likely to be due to an artifact of the method, and more likely due to the proteins being slowed by not only the viscosity of the lipid phase but also by additional interactions with other cellular components either within or to either side of the lipid bilayer (see for example reference 17). Since rhodopsin diffuses at the rate expected from the lipid phase viscosity, it appears rhodopsin is relatively free from such additional interactions.

We thank David E. Wolf for help in derivation of the equation for fluorescence photobleaching recovery. This research was supported by NIH grants 2 RO1 EY00520-11 to R. Cone and RO1-AI-14584 to M. Edidin. This is contribution number 1073 from the Department of Biology, Johns Hopkins University.

Received for publication 20 June 1980 and in revised form 8 September 1980.

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