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Upper Limit on Translational Diffusion of Visual Pigment in Intact Unfixed Barnacle Photoreceptors*

E. Almagor, P. Hillman, and B. Minke

Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem, Israel

Abstract. Translational diffusion of pigment molecules in the disc membranes of amphibian rod outer segments is in the range of 10 μ/10 s. Recently, Goldsmith and Wehner set an upper limit of 10 μ/20 min to the diffusion in isolated formaldehyde-fixed rhabdoms of crayfish. We have now used the early receptor potential (ERP) to study the diffusion in intact, unfixed barnacle photoreceptors. The ERP from a cell fully adapted to blue light (most of the pigment in the rhodopsin state) was changed by 8–22% of its maximum change when the pigment in a 30 μm spot was (almost) completely shifted to the metarhodopsin state by red laser adaptation. Further red illumination of the same spot 30 min later produced only a limited further change in the ERP (attributable to light scatter), showing that R had not migrated into the spot. It is concluded that the visual pigment diffuses by less than 30 μ/30 min.

Key words: Visual pigment – Diffusion – Photoreceptor – Membrane.

Introduction

Various approaches to the understanding of the transduction mechanism in photoreceptors have suggested that diffusion plays an important role in the transduction process. Cone (1973) and Yoshikami and Hagins (1973) have proposed an internal transmitter intervening between the photon-initiated pigment cascade and the change in plasma membrane conductance. There is evidence for Ca^{2+} being such a transmitter in vertebrate photoreceptors (Hagins and Yoshikami, 1974).

No such substance has been identified in invertebrates but there is indirect evidence for the existence of some diffusable factor that takes part in the transduction process:

a) Cone (1973) has shown that the maximum response per photon absorbed in the ventral photoreceptor of *Limulus* is too large to arise from a single ion channel,

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or even one microvillus. This observation requires a diffusion speed (of some factor) which is greater than 1 μ/100 ms.

b) Hillman et al. (1976) have studied some nonlocal interactions in the barnacle photoreceptor. They have shown that it is possible to inhibit the prolonged depolarising after-potential (PDA) from a distance greater than 0.01 μm in less than 500 ms.

The material whose diffusion is presumably responsible for the spread of the transduction process could be either an unidentified transmitter or the visual pigment itself, if its diffusion rate is high enough.

Liebman and Entine (1974) and Poo and Cone (1974) have measured the translational diffusion rate of photopigment in the disc membranes of amphibian rod outer segments. They calculated a diffusion speed of about 10 μ/10 s. This rate is high enough to account for the nonlocal processes concerning the PDA (b) and is a close approximation to the lower limit of diffusion required for explaining photon efficiency (a).

Recently, Goldsmith and Wehner (1977) set an upper limit for the diffusion rate of pigment in crayfish photoreceptors, which is about an order of magnitude less than the diffusion rate found in vertebrates. They studied isolated and formaldehyde-fixed rhabdoms. Although they used low concentrations of formaldehyde, which seem to have no effect on membrane fluidity, it is clearly desirable to study translational diffusion of the pigment in unfixed preparations.

The use of the early receptor potential (ERP) as a measure of pigment state, instead of photometry, clearly offers such a possibility (Hillman et al., 1976). The ERP is a direct manifestation of pigment transfer from one state to the other (Cone, 1964), and is easily measured in the intact, unfixed cell.

In the present experiment, therefore, the bulk of the pigment in the cell was transferred to the rhodopsin (R) state by general blue illumination. Most of the pigment in a restricted area was then converted to metarhodopsin (M) by an intense spot of red light. The ERP was then used to determine whether further red illumination of the same spot some time later converted further pigment from R to M: If so, this R must have diffused into the spot; if not, no diffusion occurred.

Methods

Balanus eburneus was obtained from Haifa, Israel. The preparation and the techniques of intracellular recording have been described previously (Hillman et al., 1973) except that the tapetum was removed in order to prevent light reflection. Penetration of the cell by the micropipette was through the tapetal side. The optical apparatus for the diffuse light stimuli has also been described previously (Hillman et al., 1973). For blue adaptation of the photoreceptor we used a Balzers K3 broad band filter with peak wavelength at 495 nm and width at half height of 45 nm. The full intensity of this blue light at the receptor was about 4×10^{14} photons $cm^{-2} \cdot s^{-1}$.

The light source for diffuse red illumination and for the red spot stimulus was a 0.5 W He-Ne laser (632.8 nm wavelength). For diffuse adaptation of the whole cell the laser beam was reflected directly onto the cell. The intensity at the photoreceptor was about 5×10^{16} photons $cm^{-2} \cdot s^{-1}$.

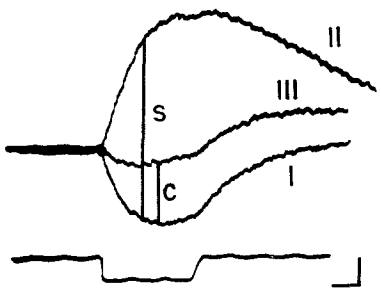

Fig. 1. Early receptor potential (ERP) responses after various adaptations. Intracellular recordings from an excised barnacle photoreceptor at 22° C in seawater saturated with CO_2 to suppress the late receptor response. White stimulus (duration indicated in lowest trace) following general blue adaptation of the cell (I), general red adaptation of the cell (II), and spot red adaptation after general blue adaptation (III). $Y = C/S$ gives the fraction of the transferable pigment transferred from the rhodopsin to the metarhodopsin pigment state. Calibrations: 10 ms, 1 mV

For illuminating a small area of the cell, the laser beam was focused by a lens onto a 70 μm single-fibre light guide. The other end of the fibre was attached to one tube of the binocular microscope, where the eyepiece had been removed. The light emerging from the fibre was focused onto the cell by the objective lens of the microscope. Attachment of the light guide to the microscope was made with the aid of a micrometer, so that the red light spot could be moved on the cell under visual control through the second tube of the microscope.

The measurements of pigment transitions are illustrated in Figure 1. This shows the ERPs induced by fixed white light stimuli after three different saturating adaptations of the receptor cell:

I. Blue diffuse adaptation – most of the pigment in the R state.

II. Red diffuse adaptation – most of the pigment in the M state.

III. Blue diffuse adaptation followed by 2 min of red spot illumination.

The relative amount (Y) of pigment transferred (R → M) by the red spot illumination is defined as the amount of R present after adaptation I minus that after III, divided by that after I minus that after II. Minke et al. (1973) have shown that the value of Y can be calculated directly from the ERPs as shown in Figure 1 by $Y = C/S$. The ratio C/S is constant during the course of the ERP. It is a function of cell adaptation only and does not depend on the stimulus inducing the ERP, as long as it is the same stimulus for the three adaptations.

Since, as a rule, the ERP is partly obscured by the late receptor potential (LRP), we suppressed the LRP by saturating the seawater with CO_2 (Atzmon, 1978).

If a cell or any part of it is initially adapted to light of a certain wavelength and then illuminated homogeneously with a light of another wavelength and constant intensity I, the population of the R state c_R should depend exponentially on the duration t of the illumination (Hochstein et al., 1978): $c_R = \exp(-t/\tau)$, where τ is a time constant inversely proportional to I.

In the red spot experiment, scatter and optical imperfection ensure that the intensity is actually a continuous function of position: $\tau = \tau(x)$. The equation would then have to be integrated over x.

We shall show, however, that the experimental curve is well approximated by the sum of two exponentials. This corresponds to a homogeneous intense disc of light plus a homogeneous weak field.

Figure 2 shows such an analysis: log c_R (relative) is plotted against t for one cell. The points are well fitted by the sum of two exponentials with $\tau = 70$ s and 29 min. Since extrapolation of the second back to zero duration gives $c_R = 0.78$, this result

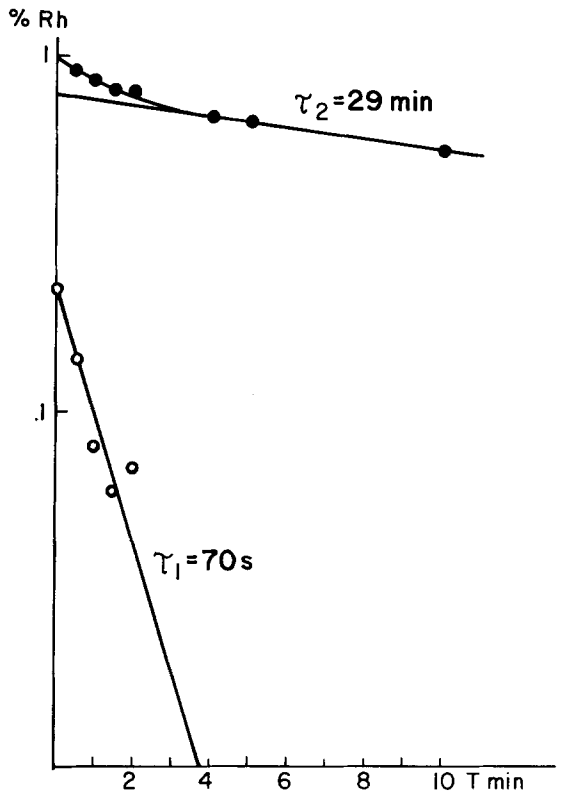

Fig. 2. Relative rhodopsin content c_R of a cell (log scale) as a function of duration of red spot illumination of a fully blue-adapted cell (filled circles). A straight line was drawn through the last three points; its extrapolated values were subtracted from the remaining points, the resulting values were plotted as open circles, and another straight line fitted to these circles. This decomposition of the curve into two exponentials corresponds to a bright uniform central spot and a uniform field of scattered light, with pigment-transfer rate constants of 70 s and 29 min respectively

corresponds to an intense disc covering 22% of the cell pigment plus a field whose intensity is 4% of that of the disc. In the other two cells used here, the disc covered 8% and 14% of the pigment. Since the rhabdomeric size in these cells is less than 60–80 μm, the disc diameter is estimated at not more than 30 μm.

Calculation of an Upper Limit for Pigment Diffusion Rate

A blue adapted photoreceptor was exposed to red spot stimuli of three different sequences:

A. A continuous stimulus for t min duration.

B. A continuous stimulus for 2 t min duration.

C. Two stimuli, of t min each, separated by a 30 min period of dark.

Using the ERP method, we measured the relative amount of pigment transfer Y induced by each stimulus. We shall refer to these values as Y_t, Y_{2t}, and Y_{t+t}, respectively.

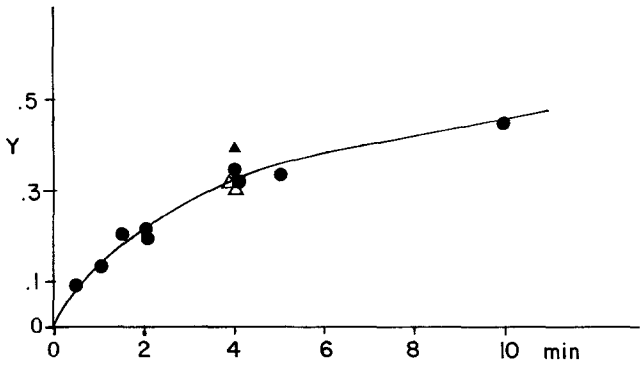

Fig. 3. The data of Figure 2 plotted as relative pigment transfer ($Y = 1 - c_R$) (linear scale) versus duration of red-spot stimulation of blue-adapted cell. The discs are the experimental points, the curve a smooth fit, the open triangles the experimental values of Y after two 2-min red spot exposures 30 min apart (Y_{t+t}) (two measurements) and the filled triangle the expected value of Y if complete diffusion of the rhodopsin had taken place during this time. The close match of the open triangles to the curve, and not to the filled triangle, indicates that no diffusion has taken place

If during the dark period the pigment had fully diffused, we would expect the first t stimulus to transfer a fraction Y_t of the pigment and the second a fraction Y_t of the remaining pigment ($1-Y_t$), so that $Y_{t+t} = Y_t(1-Y_t)$; if no appreciable diffusion had occurred, since it should then make no difference whether the two stimuli of duration t are given successively or separated in time, we would expect $Y_{t+t} = Y_{2t}$, which in general is less than $Y_t + Y_t(1 - Y_t)$.

The discs in Figure 3 show the experimental values of Y_t versus t in one cell. The expected value for Y_{t+t} if the pigment had fully diffused is shown as a filled triangle and two experimental values for Y_{t+t} as open triangles.

For the three experiments showing the largest and most stable ERP responses, values of $Y_{2t}-Y_{t+t}$ of -0.02 ± 0.04, 0.00 ± 0.03, and 0.00 ± 0.03 were observed, where the errors quoted are the standard deviations of the scatter of the results in each experiment. The average value of $Y_{2t}-Y_{t+t}$ for the three experiments is thus 0.00 ± 0.02. Similarly, values of $Y_t + Y_t(1 - Y_t) - Y_{t+t}$ of 0.06 ± 0.04, 0.08 ± 0.03, and 0.07 ± 0.03 were obtained, giving an average of 0.07 ± 0.02.

Discussion

From the observed values of $Y_{2t} - Y_{t+t}$ and $Y_t + Y_t(1 - Y_t) - Y_{t+t}$ one may calculate an upper limit to the amount of R which migrated into the spot in 30 min: One obtains a value of about 30% of its pre-adaptation content. If the membrane is considered an infinite flat plane, this corresponds to a diffusion rate of 30 μm/30 min. An approximate calculation of the geometrical effect of the membrane microvilli multiplies this by 4–120 μm/30 min.

This value is much smaller than the diffusion coefficient found in the disc membrane of amphibian rod outer segments, and is somewhat lower than the limit obtained by Goldsmith and Wehner (1977) for the diffusion of metarhodopsin in formaldehyde-fixed rhabdoms of crayfish (see Introduction). It should be noted, however,

that Goldsmith and Wehner would have detected diffusion within single microvilli while, if there is a diffusion barrier between microvilli, we can say nothing about diffusion within a microvillus.

Conclusion

The upper limit of 30 μm/30 min we have set for the diffusion rate of R molecules in the photoreceptor membrane of *Balanus eburneus* is much smaller than that measured in the disc membranes of amphibian rod outer segments and appears too low to account for the calculated efficiency of one photon in changing membrane conductance (apparently in many microcilli). Thus the need for a model for the transduction mechanism in invertebrate photoreceptors which includes an internal transmitter is strengthened. The value does *not* yet exclude the possibility that diffusion of the pigment itself may be responsible for the nonlocal inhibition of the PDA (see Introduction).

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