Spatial Properties of the Prolonged Depolarizing Afterpotential in Barnacle Photoreceptors

I. The Induction Process

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ABSTRACT In invertebrate photoreceptors, when the light stimulus results in substantial net transfer of the visual pigment from the rhodopsin (R) to the metarhodopsin (M) state, the ordinary late receptor potential (LRP) is followed by a prolonged depolarizing afterpotential (PDA). The dependence of the amplitude of the PDA on the amount of pigment conversion is strongly supralinear, and the PDA duration also depends on this amount. These observations indicate an interaction among the elements of the PDA induction process and also make possible a test of the range of this interaction. The test consists of a comparison of the PDA after localized pigment conversion, obtained by strong spot illumination, to that after weaker diffuse illumination converting a comparable total amount of pigment. The experiment was performed on the barnacle lateral eye. The effective spot size was measured by the early receptor potential (ERP), in seawater saturated with CO_2, which considerably reduced the electrical coupling between the photoreceptors. The ERP was also used to determine whether there is diffusion of R molecules into the illuminated spot. The spot illumination induced a PDA with small amplitude and long duration, while no detectable PDA was induced by the diffuse light. This indicates that the range of the PDA interaction is much smaller than the entire cell. In addition, the ERP results showed that there was no detectable diffusion of R molecules into the illuminated spot area over 30 min. This measurement, with a calculated correction for the microvillar geometry of the photoreceptor, enabled us to put an upper limit on the diffusion coefficient of the pigment molecules in the inact, unfixed barnacle photoreceptor of $D < 6 \times 10^{-9}$ cm^2 s^{-1}.

INTRODUCTION

Illumination of invertebrate photoreceptors that causes a substantial net pigment conversion from rhodopsin (R) to its dark-stable photoproduct meta-

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rhodopsin (M) results in an afterdepolarization that can be very long (up to several hours in *Drosophila*). This afterpotential is called the prolonged depolarizing afterpotential (PDA) (for reviews see Hamdorf, 1979; Hillman et al., 1983). That the PDA is a variant of the normal phototransduction process is suggested by its origin in light absorption in rhodopsin molecules (Hochstein et al., 1973), by its being composed of quantum bumps (Minke et al., 1975; Hamdorf and Razmjoo, 1979), and by its having an ionic mechanism similar to that of the late receptor potential (LRP) (H. M. Brown and Cornwall, 1975). An understanding of the PDA process should therefore be useful also in understanding the normal LRP.

The PDA has some interesting and useful properties that are not observed in the LRP (Hillman et al., 1976): (*a*) its amplitude shows an initially strongly supralinear dependence on the amount of pigment conversion from R to M; (*b*) it shows strong facilitation; (*c*) its duration increases with increasing amplitude.

The nonlinear mechanism or mechanisms responsible for these properties must have a certain range of effectiveness. The determination of this range is the main purpose of this article. A lower limit of some tens of nanometers is placed on the range by the fact that the properties described above are detectable for stimuli affecting only a few percent of the pigment (Hillman et al., 1976). The approach described here results in placing an upper limit on the range of 20–30 μm, which is much smaller than the cell size.

The approach consists of comparing the effects on the PDA amplitude and duration of localized pigment conversion obtained by illumination of a well-defined portion of the cell (spot illumination) with the effects of pigment conversion induced throughout the whole cell by diffuse light. In order to perform such experiments, we had to measure the amount of pigment converted in each case and the effective size of the spot.

In order to constrain possible mechanisms of process spread, we also determined how much pigment had diffused into the spot during the dark period after illumination. The null result places an upper limit on the diffusion rate of the visual pigment in an intact, unfixed invertebrate preparation. Goldsmith and Wehner (1977) set an upper limit for this diffusion rate in formaldehyde-fixed crayfish photoreceptors of $D = 5 \times 10^{-10}$ cm^2 s^{-1}, which is about an order of magnitude smaller than that seen in vertebrates. Later, we set an upper limit in intact unfixed barnacle photoreceptors (Almagor et al., 1979), using a technique similar to that of the present experiment but without taking into account the electrical coupling between the photoreceptors (see Methods). With the present additional observations and an improved geometrical calculation of the effects of the microvillar structure of the membrane, and with direct measurements of the electrical coupling between the cells, we have now determined the upper limit on the diffusion to be 6×10^{-9} cm^2 s^{-1}, on the assumption that the diffusion medium (the cell membrane) is homogeneous.

We used the lateral eyes of the barnacle (*Balanus eburneus*) for our experiments. In this preparation, R and M absorb maximally at 532 and 495 nm, respectively (Minke et al., 1973; Minke and Kirschfeld, 1978). From the observed spectra, we calculate that saturating red illumination after saturating blue adap-

tation shifts ~80% of the pigment from the R to the M state. The lateral eye of the barnacle is known to have a strong electrical coupling between the three photoreceptors (Shaw, 1972). We corrected the measurements of pigment shifts for this effect using direct measurements of the coupling in our experimental conditions in other cells.

METHODS

B. eburneus were obtained from Haifa, Israel, and from Gulf Specimen Co., Panacea, FL. The preparation and the technique of intracellular recording were as previously described (Hillman et al., 1973), except that the tapetum was removed to prevent light reflection. This was done with the aid of two pairs of fine forceps. First, the connective brown tissue that covers the tapetum was removed, and then the tapetum itself was gently scraped away until the photoreceptors were exposed. The photoreceptors are covered by a thin transparent layer of a connective tissue that usually does not interfere with the electrode penetration. Occasionally, the eye had to be treated for ~3 min with a solution of 1.5% protease and 1.5% collagenase (Sigma Chemical Co., St. Louis, MO) in order to soften the connective tissue. The micropipette penetration was through the tapetal side. The optical apparatus for the diffuse blue light stimuli was also as described by Hillman et al. (1973). A K3 broad-band filter (Balzers Co., Liechtenstein) with peak wavelength at 495 nm and a width at half-height of 45 nm was used. The light intensity at the photoreceptor was $\sim 10^{15}$ photons cm^{-2} s^{-1}.

The light source for diffuse red illumination and for the red spot stimulus was a 0.5-W He-Ne laser (Spectra Physics, Inc., Fountain Valley, CA) (632.8 nm wavelength). For diffuse illumination of the whole cell, the laser beam was reflected directly onto the cell. The intensity at the photoreceptor was $\sim 5 \times 10^{16}$ photons cm^{-2} s^{-1}. This light transferred half of the transferrable R to M in ~9 s.

For illumination of a small area of the cell, the laser beam was focused by a lens (× 3.5) onto a 70-μm single-fiber light guide. The other end of the fiber was attached to one tube of a binocular microscope (Unitron Co., Newton Highlands, MA), from which the eyepiece had been removed. The light emerging from the fiber was then focused onto the cell by an objective lens (UM×20, Leitz, Wetzlar, Federal Republic of Germany) of the microscope. Attachment of the light guide to the microscope was made with the aid of a micrometer (Narishige, Tokyo, Japan), so that the red light spot could be moved on the cell under visual control through the second tube of the microscope.

When the spot diameter was focused on a flat sheet and viewed through the microscope, it looked considerably smaller than a 12.5-μm-diam pinhole. However, the effective diameter of the spot on the photoreceptor, which was much larger because of light scattering within the eye, had to be estimated indirectly by the ERP method (see Hillman et al., 1976, and Results).

During the experiment, the eye was placed in a small (2 cc) chamber, which was perfused with seawater. In order to keep the red spot diameter and intensity constant throughout the experiment, the water level was maintained by sucking away the water through a vertical pipette from above. During each red spot illumination, the perfusion was stopped. When the eye was viewed through the microscope, it remained in focus, which confirmed the constancy of the red spot on the photoreceptor. This constancy was also confirmed by the reproducibility of the ERP observations.

During the ERP measurements, the experimental chamber was continuously perfused with seawater saturated with CO_2. This was shown by Atzmon (1978) to abolish the LRP without affecting the ERP. Under these conditions, the ERP can be recorded without

distortion by the LRP. ERP measurements were done at the end of each experiment, after the LRP and PDA experiments had terminated. The CO_2 in the perfusate also reduced the electrical coupling between the photoreceptors (see Results). The electrical coupling was measured by penetrating each of two of the three cells with one microelectrode. The intracellular voltage changes were measured with a homemade current injection bridge amplifier and were recorded with a tape recorder (3964A, Hewlett-Packard Co., Palo Alto, CA). The electrodes were filled with 3 M potassium acetate and had a resistance of ~15 MΩ.

RESULTS

Measurements of Pigment State After Diffuse and Spot Illumination

The state of the pigment after any given illumination was determined by measuring the ERP response to a standard white stimulus, as described by Hillman et al. (1976). The method is illustrated in Fig. 1: the upper and lower

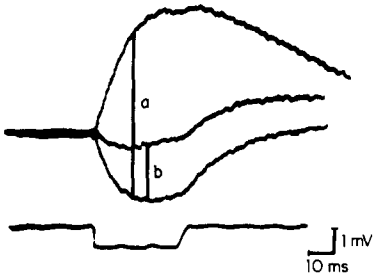

FIGURE 1. The use of the early receptor potential (ERP) to determine pigment state populations. The figure shows three superposed intracellular recordings, from a barnacle photoreceptor, of responses to the same bright white stimulus, whose duration is indicated in the separate trace at the bottom. The late receptor potential has been suppressed by the addition of CO_2 to the bathing medium. The upper and lower response traces follow saturating adaptation to red and blue lights, and correspond to maximal metarhodopsin (M) and rhodopsin (R) populations, respectively; the middle trace is for an intermediate adaptation. Minke et al. (1973) showed that the relative M population after any adaptation is given by $C_M = b/a$, measured at any particular time during the responses.

traces are the ERP responses to identical white stimuli in a cell maximally adapted to blue and red light, respectively, with maximal R and M populations. The relative concentration of pigment in the M state is defined as $C_M = ([M - [M]_{min})/([M]_{max} - [M]_{min})$, where $[M]_{min}$ and $[M]_{max}$ are the M concentrations after saturating blue and red lights, respectively. C_M can be obtained for any other adaptation from the ERP response to the same white stimulus (middle trace) by $C_M = b/a$ (Minke et al., 1973; Hillman et al., 1976). The ratio b/a is independent of the test stimulus and is constant during the course of the ERP, since for a given stimulus the ERP amplitude at any time is a linear function of the concentrations of the two pigment states.

Estimation of red spot size. If a cell is homogeneously illuminated and all rhodopsin molecules have an equal chance of absorbing light (no screening),

then C_M and $C_R = (1 - C_M)$ should approach their saturated values exponentially with the amount of illumination or the duration of illumination of constant intensity (Minke et al., 1973, 1974). A semilogarithmic plot of C_R against the duration of a diffuse red stimulus, after saturating blue adaptation, indeed shows a straight line (Fig. 2, circles).

"Spot" illumination cannot be homogeneous, because of light scattering. One therefore expects a more complex curve for C_R, as is observed (Fig. 2, triangles). However, this curve appears to be approximately decomposable into two exponentials, as shown. We interpret this to mean that the light distribution can be approximated by a small homogeneous spot of intense light together with weak homogeneous illumination of the rest of the cell. The ratio of the slopes of the two exponentials gives the ratio of the intensities inside and outside the spot, while the extrapolation of the exponentials to zero duration gives the exposed-pigment ratio. The diffused intensity was always <8% of the spot intensity, while the amount of pigment in the spot was found to be between 8 and 30% of the total. These limits are raised to 10–37% by a 1.25 correction factor for the residual electrical coupling between the cells (see below). Since the diameter of the rhabdomere was ~60–80 μm (Fahrenbach, 1965), the spot diameter was estimated to be <40 μm.

In fact, the results of Fig. 2 are not sensitive to the exact distribution of light within the central spot, and the results of Figs. 5 and 6 suggest that the light was strongly peaked at the center of the spot. Taking this into account would slightly improve the upper limits calculated below, as noted there.

The results of Fig. 2 indicate that, in addition to the localization of the spot in a small region of the cell, the pigment molecules did not diffuse into the spot during the limited time used in the above measurements. In order to extend the time range in which diffusion of the pigment could take place, we performed the next experiment.

Do the Pigment Molecules Diffuse into the Spot Area?

Using the ERP technique described above, we determined C_M after saturating spot illumination of duration $2t$, $C_M(2t)$. We compared this with C_M after two saturating illuminations each of duration t, separated by 30 min, $C_M(t, t)$. This split illumination should transfer more pigment from R to M than the consecutive illuminations if there is any diffusion into the spot.

Fig. 3 (circles) shows C_M as a function of the duration of continuous red spot illumination of a cell previously saturated with blue light. The slope of the curve decreased sharply for durations longer than 1 min, which suggests that at that duration the conversion in the spot was close to saturation and that subsequent shifts occurred mainly in the surrounding regions. The open triangles show the shift obtained after two 1-min illuminations separated by 30 min of dark ("split illumination"). The coincidence of the open triangles with the circles for 2 min duration shows that the second illumination transferred no more pigment when it was presented 30 min later than when it immediately followed the first. This suggests that no substantial diffusion of R into the spot had occurred during the 30-min period. If "complete" diffusion had occurred, the second saturating spot stimulus, presented after 30 min, would have transferred nearly as much pigment

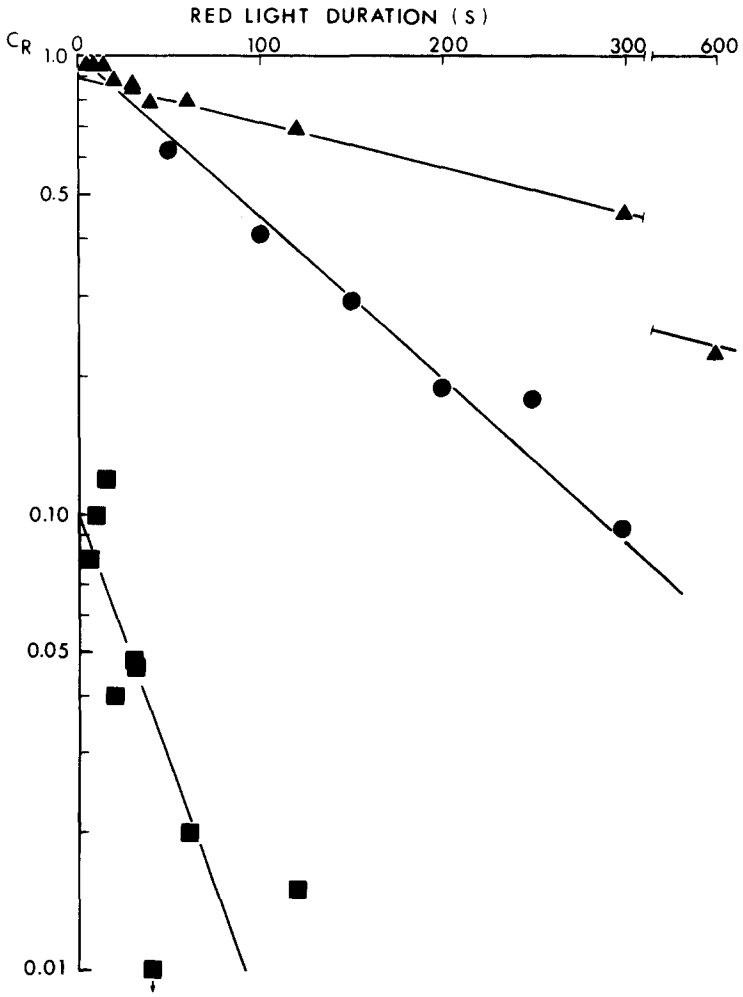

FIGURE 2. Determination of light intensity distribution during spot illumination. The relative rhodopsin population C_R (determined by ERP measurements as in Fig. 1) is plotted on a logarithmic scale against the duration of red light illumination after saturating blue adaptation of the whole cell. The values for diffuse illumination (circles) fit a straight line (single-exponential function), as expected. The results for "spot" illumination (triangles) fall on a straight line at longer durations only. The differences between the observations at shorter durations and the extrapolation of this line give the points at bottom left. These points, while very "noisy," can again be fitted by a straight line. The slopes (rate constants) of these two lines have a ratio of 16. This shows that the light intensity distribution can be approximated by a strong homogeneous small disk plus homogeneous illumination of the cell at a level ~6% of that in the spot. Extrapolation of the bottom line back to zero determines the effective disk size; here it contains ~10% of the pigment in the cell. These figures are for an isolated cell and must be corrected to 7 and 12.5% if the 0.3 electrical coupling between the cells in the ocellus is taken into account (see Fig. 4 and text).

as the first; the actual value for the shift would have been $C'_M(t, t) = C_M(t)[1 - C_M(t)] + C_M(t)$. This value is shown in Fig. 3 as a solid triangle.

We studied in the same way four different cells that were stable enough so that in each case the split illumination could be tested twice and the whole graph of the continuous illumination could be measured before and after these tasks. In all cases, there was no significant difference between the shifts of pigment by the consecutive and the split illumination. We conclude that the pigment did not diffuse appreciably into the illuminated spot.

The derivation of a quantitative upper limit for the diffusion coefficient from these observations is given in the Appendix. The result is $D < 6 \times 10^{-9}$ cm^2 s^{-1}.

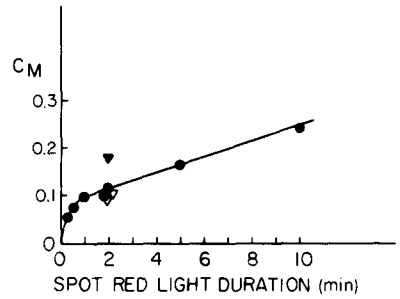

FIGURE 3. Determination of the amount of R that diffused into the illuminated spot. The ERP-determined relative M population, C_M, is plotted as a function of duration of continuous red spot illumination after saturating blue adaptation (disks). The sharp change of slope near 1 min shows that the pigment conversion in the spot is saturated by this time; the continued, but slower, conversion is due to scattered light in the rest of the cell. Each open triangle is a measurement made after two 1-min exposures separated by 30 min; their agreement with the results of a 2-min continuous exposure shows that no substantial pigment migration into the spot had taken place during the long dark period. Had "complete" diffusion taken place during this period, the expected value of C_M after the split exposure would have been much larger, as indicated by the solid triangle (see text). From this result, after correction for electrical coupling, an upper limit on the pigment diffusion coefficient of $D < 6 \times 10^{-9}$ cm^2 s^{-1} was calculated (see Appendix).

This figure takes into account diffusion into the microvillus and assumes no barrier for diffusion between the microvillar membrane and the membrane connecting the microvilli. This upper limit for D is similar to the value observed for D in vertebrate rods (Poo and Cone, 1974; Liebman and Entine, 1974; Cone and Edidin, 1981), but it is larger than the upper limit obtained by Goldsmith and Wehner (1977) in fixed crayfish photoreceptors.

The experiment of Fig. 3 also provides information about the relative size of the spot. In this particular case, 2 min of illumination shifted almost all the shiftable pigment in the spot, as evidenced by the sharp change in slope of the curve near this point. This amount corresponds to 10% of the total pigment, which means that the relative size of the spot was ~10% of the effective cell area, which was assumed to be homogeneous. According to Fahrenbach (1965), the rhabdomere in the barnacle lateral eye is mainly concentrated near the tapetal

side of the photoreceptor. On this side, it is evenly distributed over the whole cell area. This was confirmed by "surveying" the cell with the light spot. Any residual inhomogeneity would change the upper limit for D only slightly.

Effect of Electrical Coupling Between the Photoreceptors on the ERP Measurements

The photoreceptors of the lateral eye of the barnacle are strongly electrically coupled (Shaw, 1972). If one assumes total coupling between the three cells (i.e., 100% coupling coefficient), then the ERP current induced by the pigment conversion in the red spot area should spread through a membrane area that is about three times larger than that of one cell.[1] In that case, our calculations of the spot area by measuring pigment shifts would be underestimates by a factor of ~3. In the general case, the effect of a coupling coefficient p (between each pair of cells) on C_M leads to a correction factor of $3/(3 - 2p)$.

Since the electrical coupling between cells is known to be reduced under CO_2 (Giaume et al., 1980), we measured this coupling directly under the same conditions that we measured the ERP, i.e., in seawater saturated with CO_2.

Measurements of Electrical Coupling under CO_2 Conditions

Fig. 4 demonstrates one experiment (out of four) in which the electrical coupling between two cells was measured. The figure shows two voltage traces recorded in two coupled cells while current pulses were injected in one. In Fig. 4*a*, the eye was perfused with normal seawater, while in Fig. 4*b*, the seawater was saturated with CO_2. The coupling coefficient between the two cells decreased from 0.5 in Fig. 4*a* to ~0.3 in Fig. 4*b*. In the other three experiments, the coupling coefficient in CO_2 was reduced to 0.35, 0.3, and 0.27. Illuminating the cells under CO_2 conditions did not change those values.

A coupling coefficient of 0.3 increases our estimated spot size measured by pigment shifts by a factor of 1.25. The estimated relative area of the red spot is also increased by a factor of 1.25. The calculated ratio q (see Appendix) is also increased slightly. The corrected values appear in Table I.

Spatial Properties of the PDA Induction

In the experiments of Fig. 5, we compared the amplitude and duration of PDAs recorded in the same cell after two different red illuminations (see Methods): (*A*) intense spot illumination and (*B*) diffuse weaker illumination. The relative size of the spot area, ~16% of the rhabdomere area, and the amount of pigment converted were determined by the ERP as described above (Figs. 2 and 3).

There are large differences between the PDAs of *A* and *B*: (*i*) although the PDA of *A* resulted from only 5% R-to-M conversion, it was four times longer than the PDA of *B*, which resulted from 40% R-to-M conversion (40 and 10 s decay time constants, respectively). In fact, the PDA of *A* was unexpectedly nearly as long as a saturated PDA, even though it was calculated to be quite unsaturated (5% shift over 16% of the area). This observation suggests that the

[1] In fact, one of the cells is smaller than the other two, but according to Shaw (1972), the coupling between all pairs is about the same.

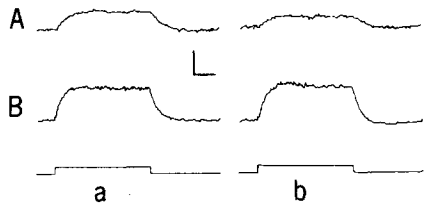

FIGURE 4. The electric coupling between two cells in regular seawater (*a*) and in seawater saturated with CO_2 (*b*). The middle traces in *a* and *b* show the voltages in cell *B*, the cell in which the current is injected. The upper traces show the voltages induced in cell *A* when a current of 25 nA was injected into *B* (timing of current shown in lower traces). The electric coupling coefficient obtained by dividing the potential in *A* by the one in *B* is ~0.5 in *a* and 0.3 in *b*. The calibration is 2 mV and 250 ms. The increase in the potential of cell *B* seen in *b* is a result of a typical increase in membrane resistance caused by the CO_2.

effective size of the spot is much less than 16%, and its size is therefore probably <20 μm. (The fact that the PDA amplitude of Fig. 6*A*, which was recorded from the same cell, does not increase after the first stimulus, which is identical to that of Fig. 5*A*, supports this idea.) (*ii*) In spite of its long duration, the PDA of trace *A* has an initial amplitude that is four times smaller than that of the shorter PDA of trace *B* (note different amplitude scales for *A* and *B*). The amplitude of the LRP is also considerably smaller in trace *A* compared with trace *B*, which confirms that only part of the cell was illuminated by the spot.

Fig. 6 shows PDAs recorded from the same cell as Fig. 5 (with reduced gain), for repeated brief spot and diffuse illumination, respectively. Each spot stimulus shifted roughly the same amount of pigment as each diffuse stimulus (5%), but a single spot stimulus (Fig. 6*A*) resulted in a substantial PDA (see Fig. 5, where

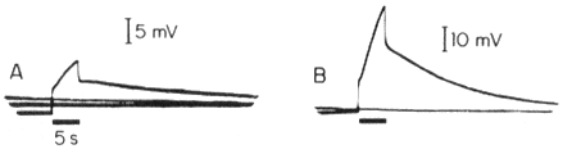

FIGURE 5. A comparison of prolonged depolarizing afterpotentials (PDAs) induced by "spot" and "diffuse" stimuli. In all cases, the cell had been adapted to saturating blue illumination (leaving the pigment largely in the R state), followed by 5 min of darkness. All stimuli were red (transferring the pigment to the M state) and of 5 s duration (marked by bars). *A* shows four and *B* shows two successive oscilloscope sweeps. The two lower traces in *A* are indistinguishable. Trace *A* is for "spot" illumination, which in this cell was found by the ERP technique of Fig. 2 to cover 16% of the cell pigment; the light intensity outside the spot was 16 times weaker. In *B*, the stimulus covered the entire cell. The individual stimuli in *A* and *B* transferred ~5 and 40% of the transferable pigment from R to M, respectively. A comparison of *A* and *B* shows that even though the amplitude of the "diffuse" PDA is much larger, because much more pigment is transferred, it has a shorter decay time than the "spot" PDA; normally, decay time increases monotonically with amplitude.

a similar PDA was recorded at higher gain and without interruption by further stimuli), which increased no further with further stimulation, while the diffuse stimulus elicited a noticeable PDA only after the ninth stimulus (Fig. 6*B*). (The hyperpolarization after the earlier stimuli presumably arises from ion pump electrogenicity [Koike et al., 1971; J. E. Brown and Lisman, 1972].) This PDA then increased after further diffuse stimuli, to a much larger amplitude than the saturated spot PDA. The observation that spot stimulation resulted in long, small PDAs was confirmed in the 11 other cells that were examined. In two cells, we also checked that diffuse stimuli transferring the same amount of pigment induced no detectable PDA.

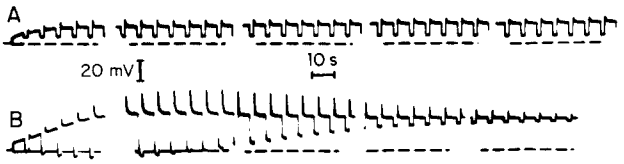

FIGURE 6. These traces were recorded from the same cell as that in Fig. 5 and with the same experimental paradigm, except that the individual stimuli, which transferred ~5% of the pigment by spot illumination (*A*) and diffuse illumination (*B*), were repeated many times. Trace *A* (like trace *A* in Fig. 5, which was recorded in the same cell) shows that a 5% pigment transfer, when concentrated in a spot, was enough to produce an appreciable PDA in this cell. In contrast, in the case of the diffuse stimuli of trace *B* in the same cell, no appreciable PDA appeared until well over 30% of the pigment was transferred (nine stimuli). The hyperpolarization following the earlier stimuli was presumably due to ion pump electrogenicity.

DISCUSSION

The fact that a localized PDA can have a small amplitude and a long duration shows that the range of the interaction responsible for the nonlinearities of the PDA induction is short compared with the localization; i.e., it is <40 μm. A previous lower limit of some tens of nanometers had been established (Hillman et al., 1976), but if pigment molecule aggregation occurs as is suggested below, both observations are consistent with confinement within the pigment aggregate.

The photoreceptors in the lateral eye of the barnacle are known to be strongly electrically coupled (up to 0.8 coupling coefficient at resting potential; Shaw, 1972). We confirmed this fact in our experiments. The ERP measurements were carried out on cells in CO_2-saturated seawater (see Methods). Such conditions uncouple electrically coupled cells in other invertebrate preparations (Giaume et al., 1980). We found by direct measurement of the coupling coefficient in the barnacle eye that the coupling ratio was reduced to ~30% under CO_2 conditions. Taking this coupling into account increases our estimates of the spot area by a factor of 1.25, and the diffusion constant by ~1.4.

The electrical coupling of the cells could also change the differences between the duration and amplitude of the PDA induced by spot and by diffuse illumination. If the PDA duration and amplitude are similar in the coupled cells—and our observations (not shown) suggest that this is generally true—then the

coupling would reduce the amplitude of the spot PDA without greatly changing its duration, but would not change these parameters in the diffuse PDA, since the diffuse light covers all the coupled cells. A correction for this effect would therefore only strengthen our conclusions. Another consideration is the possible influence of a time-varying coupling on the time course of the spot PDA. Since light reduces coupling (Shaw, 1972), the coupling would presumably increase during the course of the spot PDA, shortening its duration. Since a correction for this effect increases the difference between the "true" spot and diffuse PDAs, our conclusion is again only strengthened.

What might be responsible for the nonzero but limited range of the PDA interaction? Recent experiments have uncovered mechanisms by which the visual pigment is inactivated after illumination. In vertebrate rods, the bleached rhodopsin molecule may be inactivated by rhodopsin kinase and the 48,000 protein (Liebman and Pugh, 1980; Sitaramayya and Liebman, 1983*a*, *b*; Kuhn, 1984). In the fly, pigment conversion initiates phosphorylation (and dephosphorylation) of the photopigment (Paulsen and Dentrop, 1984). We hypothesize that a PDA may arise from inhibition of inactivation, and that this inhibition may be due to neighboring active molecules forming an inactivation-resistant complex. (Such a complex resembles the long-lived high-energy M state of the model of Hamdorf and Razmjoo, 1977.) If this complex is more efficient in changing conductance than the isolated molecules, the supralinear dependence of the initial PDA amplitude on light amount is predicted. The proposal that the PDA is a pigment process is supported by the observations of Blumenfeld et al. (1985) in cell-free membrane preparations of *Musca* eyes that the PDA phenomenology is present already in the GTPase activity. Since the G-protein responsible for the GTPase activity is itself activatable directly by the photopigment (Saibil and Michel-Villaz, 1984), the PDA induction processes apparently operate at the pigment level.

Our present observation that the initial amplitude of the spot PDA is much higher than that of the diffuse PDA for the same pigment conversion is consistent with the idea of such complexes as the source of the PDA since the diffusion of the pigment molecules, and so surely of the complexes, is strongly restricted (see above).

The observation that the spot PDA is also of much longer duration than the diffuse PDA can have at least two explanations. Within the model of complexes, one could suggest that the larger the complex, the longer it lives. Alternatively, the inactivating material may be locally exhausted by saturating illumination. Some support for the existence of such a material may arise from the observation that the GTPase activity associated with the PDA declines much more rapidly in intact *Musca* photoreceptors than in washed membrane preparations (Blumenfeld et al., 1985). Our results require that such an inactivating material (kinase?) have a limited diffusion rate or range in the cell.

An alternative model is that of Hochstein et al. (1973), according to which the PDA is due to separate excitor molecules activated by the R-to-M pigment transfer. If the same properties are ascribed to the excitor as to the activated pigment molecules in the preceding model, the same predictions arise. However, there is no physiological evidence for the existence of any such excitor.

Our previously published value for the diffusion coefficient of visual pigment in unfixed barnacle photoreceptor membranes (Almagor et al., 1979) was in error. The new value given here no longer requires the diffusion in this preparation to be slower than in the vertebrate.

APPENDIX

Calculation of an Upper Limit for the Pigment Diffusion Coefficient

A cell is illuminated with a homogeneous disk of light for a time t or a time $2t$, or twice at the same place for a time t with a dark interval in between. The observed fractions of the pigment in the M state are $C_M(t)$, $C_M(2t)$, and $C_M(t, t)$, respectively.

Suppose that during the dark period, the spot area was enriched by a fraction q of the maximal number of R molecules that could enter the spot. This maximum corresponds to reaching complete homogeneity of the R/M ratio over the rhabdomere.

If $q = 0$, then

$$C_M(t, t) = C_M(2t). \tag{1}$$

If $q = 1$, then

$$C_M(t, t) = C_M(t)[1 - C_M(t)] + C_M(t) \equiv C'_M(t, t). \tag{2}$$

Since in general the rate of pigment shift (R to M) is linear with the amount of R present, $C_M(t, t)$ is a linear function of q, so

$$C_M(t, t) = C_M(2t) + q[C'_M(t, t) - C_M(2t)] \tag{3}$$

or

$$q = \frac{C_M(t, t) - C_M(2t)}{C'_M(t, t) - C_M(2t)}. \tag{4}$$

Table I displays the observed values of the various parameters of this equation: the duration t in minutes; the effective amount a of pigment in the disk relative to that in the whole cell; the observed values of $C_M(t)$, $C_M(2t)$, and $C_M(t, t)$; and the calculated value of $C'_M(t, t)$. A 90% confidence interval is indicated for each pigment shift. These intervals were deduced by fitting a sum of two exponential curves to the whole series of pigment shifts at various durations of illumination as in Fig. 2. The procedure for this calculation is as follows.

To calculate a 90% confidence interval for q, we substituted in Eq. 4 the values of the various parameters with their 90% confidence limits added or subtracted so as to give the largest value of q or the smallest value of q. This is very much a worst-case calculation, since it corresponds to a complete and appropriate correlation among the errors of the parameters in the equation. The calculated intervals from q appear in Table I. In the last column, the table shows the maximal values for q corrected for the 30% electrical coupling between the cells. The correction was obtained by multiplying each of the pigment shifts and their confidence intervals by the factor 1.25 as explained earlier.

The mean q values in all cases are small and do not deviate significantly from zero, which shows that a zero diffusion constant could not be ruled out by our experiment. In the last three cells, the q^{max} value is <1, which allows the calculation of an upper limit on the diffusion constant, D. D was calculated for each of the cells as follows.

We first consider the cell membrane to be a plane sheet. The initial conditions are approximated by a small disk with a homogeneous low concentration of pigment. The

solution for such conditions is given, for example, by Crank (1956). For small q (as in the last three cells), the solution is $D = \pi(dq)^2/8\tau$, where τ is the time available for diffusion (here 30 min) and d is the disk diameter, which is the square root of the fraction of the pigment in the spot times 80 μM. The calculated values of D appear in Table I.

To place an upper limit on the diffusion coefficient, one might in principle use the lowest upper limit of all the values of D. However, in order not to rely on a single cell, we take the average of D in these relevant cells, which is 3×10^{-10} cm^2 s^{-1}.

To correct this result for the microvillar structure, we performed a simulation computer calculation. The calculation assumes that there is no barrier for diffusion between the microvillar membrane and the membrane connecting the microvilli and that the membranes are similar.

A Monte Carlo calculation of a point moving randomly in small steps was carried out for two different surfaces. One was a flat plane and the other was a surface of densely packed cylinders protruding from a plane, resembling the microvillar structure. The

TABLE I

Upper Limits on the Pigment Diffusion Coefficient

Cell	t	a	$C_M(t)$	$C_M(2t)$	$C_M(t, t)$	$C'_M(t, t)$	q	q^{max}	$D <$
1	0.5	0.37	0.27±0.02	0.36±0.02	0.39±0.04	0.47±0.03	0.3+0.6 −0.8	1.1	∞
2	1	0.10	0.095±0.004	0.113±0.004	0.11±0.007	0.18±0.007	−0.04+0.16 −0.2	0.12	2×10^{-11}
3	2	0.28	0.228±0.006	0.313±0.006	0.32±0.01	0.404±0.009	0.08+0.22 −0.24	0.35	5×10^{-10}
4	2	0.22	0.18±0.02	0.20±0.02	0.18±0.04	0.33±0.04	−0.17+0.51 −1.0	0.36	4×10^{-10}

The table shows the observed and calculated values and standard deviations of the parameters participating in the calculation of q, the fraction of the R molecules that could have entered the spot of light between illuminations that actually did so. t is the duration in minutes of each illumination; a is the fraction of the pigment within the central illuminated disk; $C_M(t)$, $C_M(2t)$, and $C_M(t, t)$ are the observed values of the M pigment fraction after single, double, and split illuminations; $C'_M(t, t)$ is the M fraction, which should have been observed if full diffusion had taken place; q is derived from the difference between $C_M(t, t)$ and $C'_M(t, t)$ (Eq. 4 of Appendix); q^{max} is the maximal value q could have, with 90% confidence; and $D <$ is the upper limit on the pigment diffusion coefficient in square centimeters per second, derived from the values of q^{max} using the equation in the Appendix text. A final limit on D of $D < 3 \times 10^{-10}$ cm^2 s^{-1} is obtained by averaging the limits obtained in the last three cells. This is corrected to $D < 6 \times 10^{-9}$ cm^2 s^{-1} by taking into account the microvillar geometry (Appendix text).

height of the cylinders was 10 times their diameter, and the calculation step was 0.01 of this diameter. We counted the number of steps taken by the point to travel a lateral distance of four cylinder diameters. The point could move either on the cylinder surface or on the connecting basal plane. The random walk calculation was carried out as follows. The point was started at a position 0,0 in between cylinders. It was then displaced a distance 0.01 in a random direction. If at the end of the step the point was still on an area in between cylinders, it then made a new random step. If, on the other hand, it had entered an area occupied by a cylinder, its path was continued on the cylinder surface with the same angle with respect to the rim as the approach angle at that point. Thus, the angle between the path of the point and the radius of the cylinder base was transferred to an angle between the cylinder axis and the path of the point on its surface, while the length of its path was conserved. A similar procedure was performed whenever the point went out of the cylinder at its top or at its bottom.

The result of this calculation is that the number of steps required to travel the fixed distance is greater by a factor of ~20 in the microvillar case. Thus, if there is no barrier for diffusion between the microvilli, the diffusion coefficient of rhodopsin must be smaller than $\sim 6 \times 10^{-9}$ cm^2 s^{-1}.

The errors of the various pigment shifts were calculated in the following way. First, a two-step linear regression was carried out on the $\log(C_R)$ curve, as in Fig. 2. This curve-fitting involves three parameters. We have used the regression curve to recalculate the expected values of $C_M(t)$ and $C_M(2t)$ and their standard deviations by $t_{0.05[n-3]}$ of the t distribution. n is the number of observation points on the graph. (For the technique, see Sohal and Rohlf, 1981.)

By substitution in Eq. 2, a similar interval for $C'_M(t, t)$ was calculated. Finally, we assume that the split illumination shift $C_M(t, t)$ has the same origin of variance as $C_M(t)$, but since it does not necessarily belong to the same population, its error is not reduced by there being n observations. This increases the confidence interval of $C_M(t, t)$ by a factor $n - 3$.

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