

Spatial Properties of the Prolonged Depolarizing Afterpotential in Barnacle Photoreceptors

II. Antagonistic Interactions

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ABSTRACT In the preceding article, we investigated the spatial properties of the induction of the prolonged depolarizing afterpotential (PDA) by shifting visual pigment from the rhodopsin (R) to the metarhodopsin (M) state in the barnacle photoreceptor. In this work, we have studied the ranges within the cell of the antagonistic effects on the PDA of M-to-R transfer. When this transfer occurs during a PDA, it depresses the PDA; when it precedes PDA induction, it impedes that induction (“anti-PDA”). These ranges were previously shown (by a statistical technique) to be at least a few tens of nanometers within a half-second ($D > 10^{-13} \text{ cm}^2 \text{ s}^{-1}$). We now demonstrate, with local illumination techniques in which a PDA was induced in one side of the cell and PDA depression or anti-PDA was induced in the other side, that both ranges are much smaller than the cell diameter ($\sim 100 \mu\text{m}$) within 30 s ($D < 10^{-6}$). We further show, using a less direct but shorter-range technique involving colored polarized light, that the interaction of the PDA with the anti-PDA is restricted to less than $\sim 6 \mu\text{m}$ ($D < 6 \times 10^{-9}$). This figure is quite low and suggests that the interaction may be confined to the pigment molecules, possibly in a complex of the type suggested in the preceding article.

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 membrane conductance. Cone showed that the maximum response per photon absorbed in the ventral photoreceptor of *Limulus* is too large to arise from a single ion channel or even from one microvillus.

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Considerations based on this observation call for diffusion at a speed $>1 \mu\text{m}/100 \text{ ms}$, which corresponds to a diffusion coefficient $D > 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ for unimpeded diffusion.

A similar diffusion rate is suggested by the observation of Brown and Coles (1979) that, in a dark-adapted *Limulus* cell, absorption of fewer than 1,000 photons by the pigment molecules is sufficient to cause saturation of the membrane conductance; thus, each photon influences at least 1/1,000 of the membrane area, or $\sim 1 \mu\text{m}^2$, within 0.2 s.

In invertebrates, the transduction process manifests itself in two antagonistic effects (Hochstein et al., 1973) that arise from the interconversion by light of the visual pigment between its two stable states, rhodopsin (R) and metarhodopsin (M). The R-to-M shift induces the normal late receptor potential (LRP), but when the shift is large and unaccompanied by an M-to-R shift, the LRP is followed by a prolonged depolarizing afterpotential (PDA). The reverse shift, M to R, when it occurs after the decay of a maximal PDA, is followed by a period during which PDA induction is impeded (the "anti-PDA"), or, when it is initiated during a PDA, by a depression of the PDA. The PDA phenomenology is summarized in Hillman et al. (1977). In barnacles, the R and M absorption spectra are peaked near 532 and 495 nm, respectively (Minke et al., 1973; Minke and Kirschfeld, 1978), so that blue adaptation maximizes the R population and red light maximizes the M population.

Hillman et al. (1976) showed that the PDA can be depressed or its induction impeded even when the R-to-M and M-to-R shifts take place in sets of pigment molecules that are statistically very different. This was shown by using sufficiently weak light stimuli that only a fairly small fraction of the pigment was affected by each stimulus. In that way, $\sim 10\%$ of the molecules participated in inducing a PDA, and 10% in depressing the PDA or inducing the anti-PDA. The overlap of these two sets of pigment molecules was only 10% of each set (i.e., 1% of the total pigment). The fact that the PDA was nevertheless fully depressed suggests that the interaction includes some factor that diffuses a distance of at least some tens of nanometers in $<0.5 \text{ s}$ (the period between the light stimuli that induced and depressed the PDA or induced the anti-PDA). Alternatively, if the PDA induction involves the formation of a pigment complex of at least ten molecules (see preceding article), the "nonlocal" PDA depression may arise from a disruption of this complex.

To constrain further the possible mechanisms of the PDA interactions, we report experiments to determine or place an upper limit on the spread, from the point of photon absorption, of the mechanism responsible for the interaction(s) of the PDA with the anti-PDA and PDA depression. As modeled by Hochstein et al. (1973), these two effects arise from the same "inhibitory" process. However, Hamdorf and Razmjoo (1977, 1979) model them as entirely distinct processes, so for present purposes we treat them separately.

The present approach is the induction of the R-to-M and M-to-R transfers in separate portions of the cell. If the range of the antagonistic interaction is large compared with the separation of the illuminated areas, spatial separation will not affect the outcome; if the range is small compared with this separation, the PDA

should be unaffected by the induction of the M-to-R transfer except by light scatter.

The outcome of these experiments will give us some indication of whether the processes underlying the PDA induction and depression involve the production of highly diffusible substances (such as the release of internal transmitter) or whether they are restricted in their spread, which would suggest localization, possibly within the microvillar membrane, and perhaps even to neighboring pigment molecules (see preceding article).

METHODS

Balanus eburneus were obtained from Haifa, Israel. 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 and micropipette penetration was through the tapetal side. In part of the experiments, we also used the median eye of the giant barnacle *Balanus nubilus*, which were obtained from Seattle, WA. The optical apparatus for the diffuse light stimuli was also as described by Hillman et al. (1973). The light source was either a 150-W xenon arc lamp (XBO, Carl Zeiss, Inc., New York) or a 100-W quartz-iodide lamp. For blue and red adaptation, respectively, we used K3 and K5 broadband filters (Balzers Co., Liechtenstein) with peak wavelengths at 495 and 600 nm and widths at half-height of 45 and 50 nm, respectively. The lights and filters were chosen for maximum R-to-M or M-to-R pigment population ratios, subject to a reasonable rate of approach to that maximum (for instance, the M/R equilibrium ratio increases with wavelength above 600 nm, but the total absorption decreases rapidly, so that approach to equilibrium would require too long an exposure).

For stimulation of the two halves of the cell by two different wavelengths, the light from the xenon lamp was focused into a parallel beam. A double filter-holder rotatable by 180° was mounted so that half of the parallel beam passed through one filter (blue or red) and the other half through the other filter (red or blue). A black strip separated the filters to minimize overlap of the two colors at the cell. Either filter could be blocked by a cap. The beam was focused symmetrically onto the end of an image guide, whose other end was attached to one tube of the binocular microscope, as described in the preceding article. The image on the cell contained two 100- μm bands of light separated by a nominal 5- μm band of darkness. At the beginning of each experiment, the image was moved on the cell until it gave about the same unsaturated intracellular response to the red stimulus in the two positions of the filter holder.

Early receptor potential (ERP) measurements were used, as described in the preceding article, to calibrate the pigment effects of each color of light.

For the polarized-light experiment, the unpolarized laser beam (see preceding article) passed through either of two perpendicularly oriented, linearly polarizing filters. The filters were exchanged by a hydraulic piston.

All experiments were repeated at least six times in each of at least four cells.

Electron Microscopy

The eyes were fixed in the following solution for 1 h: 2.5% glutaraldehyde, 0.05% MgCl_2 , 3.5% sucrose, and 0.1 M buffer cacodylate. Post-fixation was with 2% OsO_4 for 1 h. The preparation was dehydrated in ethanol and embedded in Spurr (Electron Microscopy Sciences, Fort Washington, PA). Sections of ~ 600 Å were cut on an ultratome, picked up on a grid of 200 mesh, and stained with lead citrate. Electron micrographs were taken with a Jeol (Tokyo, Japan) 100CX electron microscope.

RESULTS

Range of PDA Depression Interaction: Half-Cell Technique

M-to-R conversion of pigment in one set of molecules can depress a PDA induced by R-to-M conversion in a largely different set of pigment molecules (Hillman et al., 1976). Is the range of this interaction as big as the whole cell? To answer this, we induced a PDA by R-to-M transfer in one half of the cell and determined the effect of a subsequent and comparable M-to-R transfer in the other half. The results are shown in Fig. 1.

We first balanced the illumination such that neutral stimuli (i.e., red light after red adaptation or blue light after blue adaptation, which does not transfer net pigment between the two states) induced equal LRPs in both halves of the cell, as seen in Fig. 1A. Fig. 1B shows that the PDAs induced by R-to-M stimuli obtained by two red pulses were roughly equal. (Stimulation of the second half of the cell appeared always to give a slightly larger LRP, in whichever order the two halves were illuminated. This apparent LRP facilitation effect of R-to-M stimuli probably arose from an LRP induced locally by scattered light; a much weaker stimulus was needed to induce LRP facilitation than to induce PDA depression.)

Fig. 1C calibrates the amount of blue-induced M-to-R transfer needed to depress the PDA when presented to the same side of the cell as the red PDA-inducing stimulus. Two 1-s blue stimuli sufficed to depress the PDA fully. When the blue M-to-R stimulus was presented to the other side of the cell, however, very little depressing effect was seen, even after 10 stimuli (Fig. 1D). To check whether the small residual PDA depression seen in Fig. 1D was ascribable to a scattering of the blue light into the PDA half of the cell rather than to a spread of the processes in the cell, the light-scattering control shown in Fig. 1E was carried out. Here the side that was stimulated with blue light had previously been adapted to blue instead of red light, so that no net M-to-R transfer took place (the blue stimulus was "neutral"). The blue stimulus nevertheless had about the same depressing effect on the PDA as the M-to-R blue stimulus of Fig. 1D. This depression therefore probably arose entirely from light scattering. These observations were fully replicated in four other cells. We conclude that the range of the interaction of the PDA with the PDA depression was much smaller than the cell size in 30 s.

A reservation to this conclusion could arise from the fact that the lateral eye of the barnacle is composed of three photoreceptors that are electrically coupled (Shaw, 1972; see also the preceding article). In principle, it is therefore possible that the failure of the blue illumination in Fig. 1D to influence the PDA is due to its falling on a different, though coupled, cell. However, the coupling is greatly reduced during illumination (Shaw 1972), perhaps because of an increased Ca^{2+} concentration in the cell. Since this increase is maintained during the PDA (in the fly, Kirschfeld and Vogt, 1980; Stavenga et al., 1975), the cells are probably too weakly coupled during the PDA to be consistent with our observations, which were repeated in four cells.

To minimize this reservation, however, we repeated the experiments of Fig. 1

in the median photoreceptors of the giant barnacle (*B. nubilus*). In this preparation, there is no measurable electrical coupling between the photoreceptors (Hudspeth and Stuart, 1977). Fig. 2, *B* and *D*, shows that when the blue stimuli (right column) were given to the same side in which the PDA was induced by red light (left column), PDA depression was observed. However, when the same

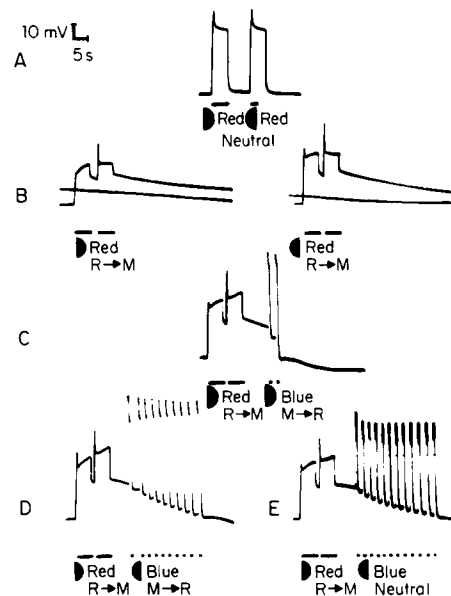


FIGURE 1. Can the PDA induced by R-to-M pigment transfer in one half of the cell be depressed by M-to-R transfer in the other half? All traces are intracellular records from photoreceptors of the lateral ocellus of *B. eburneus*. The stimulus duration and color are indicated below each trace, together with the type of pigment transfer induced and the half of the cell illuminated (left or right). *A* and *B* show that the pigment absorption is balanced between the two halves. In *A*, the prior adaptation was red, so that the stimuli were "neutral" and induced late receptor potentials (LRPs) only. In *B*, the prior adaptation was blue, so PDAs were induced that were nearly equal. (The second LRP is apparently facilitated here by scattered light from the first stimulus; R-to-M lights tend to facilitate more than neutral lights [Hanani and Hillman, 1976].) *C* shows that two blue stimuli, when presented to the same half of the (blue-adapted) cell as the red PDA stimulus, and after it, were sufficient to suppress the PDA completely. When they were presented to the opposite side (*D*), only a small effect was seen. Here the right side of the cell had been blue-adapted and the left had been red-adapted. When the whole cell was blue-adapted, the blue stimulus was neutral and induced no M-to-R transfer. However, as seen in *E*, it still induced a small depression of the PDA comparable to that seen in *D*. This suggests that the depression was due to scattered light and not to the spread of the depressing interaction, so the range of this interaction between the PDA and the PDA-depressing process was clearly smaller than the average distance between the two halves of the cell, $\sim 50 \mu\text{m}$.

M-to-R blue stimuli were given to the opposite side of the cell (Fig. 2C), if it had been previously red-adapted, a much smaller PDA depression was observed, which confirmed the previous conclusion. The effect was less striking here than in *B. eburneus* because the "neutral" PDA that occurred in both was cancelled in *B. eburneus* by a much larger ion pump electrogenicity. Fig. 2A shows the unmolested PDA in the same cell.

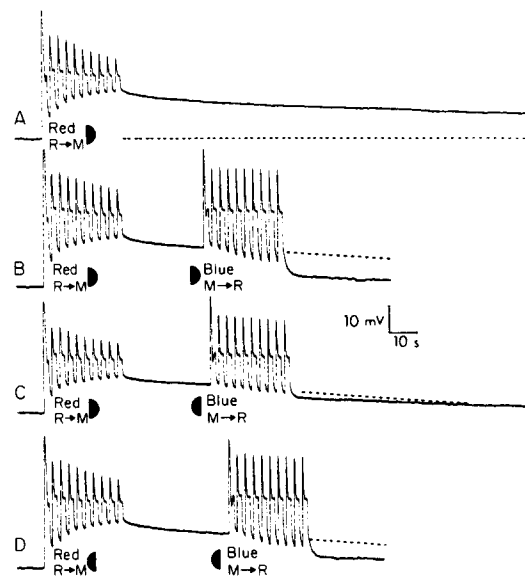


FIGURE 2. The same experiment as in Fig. 1 except that the median eye of the giant barnacle *B. nubilus* was used, in which there is no electrical coupling among the photoreceptors. *B* and *D* show that when the PDA-inducing red light and the PDA-depressing blue light were given to the same side of the cell, a clear PDA depression was observed. When the PDA-inducing stimulus was given to one half of the cell and the PDA-depressing stimulus was given to the other (*C*), the PDA depression was much reduced, the residual effect being ascribable to light scatter. The fact that the whole response is reduced in *C*, even though it was recorded between *A* and *B*, apparently arises from the difference in adaptation conditions. The dashed lines in *B–D* show the time courses the PDAs would have had without the blue stimuli as judged from *A*, which was recorded under similar conditions.

Range of Anti-PDA Interaction: Half-Cell Technique

M-to-R pigment transfer impedes later induction of a PDA by R-to-M transfer ("anti-PDA"). Does this interaction extend throughout the cell? We examined this question with the following procedure (Fig. 3). We blue-adapted the whole cell (maximal pigment in R), and then we gave a red stimulus to the right half of the cell. The resulting unmolested PDA is shown in Fig. 3A. After the complete decay of this PDA, we gave to the red-adapted right side a strong blue light that shifted the pigment from M to R (Fig. 3B, left trace), and 1 min later we shifted the pigment back from R to M in the same side with the same red stimulus as in

A. The resulting PDA of *B* (right) was considerably smaller and shorter than that of *A* because of the induction of the anti-PDA by the preceding M-to-R blue stimulus. The difference in the PDAs of *A* and *B* (right) was much larger when, instead of a 1-min dark interval between the blue and red light, we gave a dark interval of ≤ 10 s (not shown). This is because the anti-PDA decays with time (see below). We gave a 1-min dark interval in order to have a direct comparison with

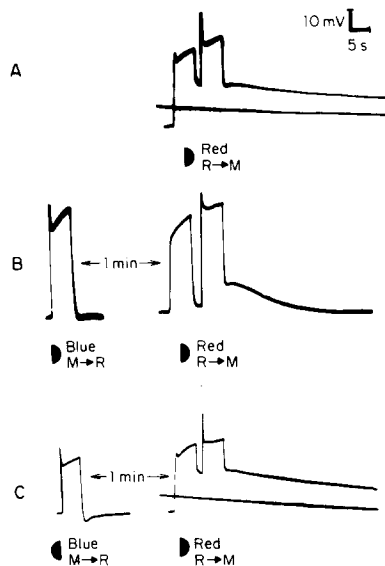


FIGURE 3. Is PDA induction by R-to-M transfer in one half of the cell impeded by prior M-to-R transfer (“anti-PDA”) in the other half? In *A* and *C*, the left half of the cell had been adapted to red light and the right to blue; in *B*, vice versa. In the control (*A*), no anti-PDA induction preceded the PDA induction. *B* shows the effect on the PDA of inducing an anti-PDA in the same half of the cell as the PDA—there was a strong reduction in both the amplitude and duration. In contrast, when the anti-PDA was induced in the opposite half of the cell, as in *C*, the PDA was not noticeably different from that in *A*. The range of the interaction of the anti-PDA with the PDA must therefore be less than ~ 50 μm . The enhanced blue LRP in *B* may arise from an imbalance in the illumination of the two halves of the cell, and the enhanced red LRPs may arise from facilitation by the preceding illumination of the same half of the cell.

the next experiment, in which we also interposed a 1-min interval between the PDA and anti-PDA induction to allow for a hypothetical diffusible substance to diffuse from one side of the cell to the other (Fig. 3*C*). In Fig. 3*C*, the left side of the cell had been red-adapted and the right side had been blue-adapted as in *A*. After the decay of the PDA in the left side, a blue M-to-R stimulus was given to that side, which induced an anti-PDA (left trace). 1 min later, two successive red pulses were given to the blue-adapted left side, which induced a PDA very similar to that of *A* (right). Fig. 3*C* thus demonstrates that if an anti-PDA is induced in one half of the cell and a PDA is induced in the other half, the PDA

is far less affected than in the case where the PDA and anti-PDA are induced in the same half (Fig. 3*B*). The residual effect can probably be attributed to light scattering (see preceding article). We conclude that the range of interaction of the anti-PDA with the PDA was also much smaller than the cell size.

Range of Anti-PDA Process: Polarized Light Technique

To investigate shorter ranges for the antagonistic interactions, one must shorten the distance between the cell volumes in which the PDA and anti-PDA are induced. Because of light scattering and because the induction of a measurable PDA requires the transfer of an appreciable fraction of the pigment in a cell, it is difficult to reach smaller distances by using spots of light. Instead, we exploited the unusual microvillar morphology of barnacle photoreceptors.

In these cells, the microvilli are composed of outfoldings of the membranes as multiple "fingers" or dendrites protruding from the main cell body (Fahrenbach, 1965). Fig. 4 shows a typical electron microscope cross-section of one of these dendrites. One can see that the microvilli are grouped into blocks of parallel microvilli. The blocks are of up to a few microns in diameter, and in each, the long axis of the microvilli is roughly radial to the dendrite axis. This structure means that microvilli a few microns apart tend to have perpendicular orientations.

The second base of the technique is the fact that microvilli have a nonunity dichroic ratio; that is, they have different absorption coefficients for light polarized along and perpendicular to their axes. Although there is some disagreement about the actual value of the ratio in different preparations, it is accepted that the pigment molecules are confined within the plane of the membrane, and this leads directly to a minimum dichroic ratio of 2 (Moody and Parriss, 1961).

The technique involves the induction of the anti-PDA and PDA by polarized light. When the orientations of the stimulus polarizations are parallel, the affected pigment populations are coextensive; when they are perpendicular, there is an average separation between the two populations, and we can determine the anti-PDA interaction range relative to this average separation. The average separation is quantitatively estimated in Appendix I.

Fig. 5 and its legend give the experimental procedure for carrying out the "parallel" and "perpendicular" experiments. The final stimuli were identical in both cases: an unpolarized anti-PDA-inducing (M-to-R) blue stimulus was followed by a PDA-inducing (R-to-M) "horizontally" polarized red stimulus. In the parallel case (Fig. 5*A*), the blue stimulus (III) induced an anti-PDA in the "horizontal" population only, since only that population was put into the M state by prior "horizontal" red illumination (II). In the "perpendicular" case (Fig. 5*B*), the situation was similar except that a prior "vertical" red illumination (II) was given instead of a prior "horizontal" red light.

The unpolarized blue stimuli were always saturating for all the pigments. In the parallel case, the polarized red prior adaptation (II in Fig. 5*A*) and the PDA-inducing stimuli (IV in Fig. 5*A*) were identical, so equal but opposite pigment transfers would be involved in the PDA and anti-PDA inductions. In the perpendicular case, however, the two red stimuli had perpendicular orientations, so

that the two affected populations were not necessarily identical. To check for this, the experiment was always performed a second time in the same cell, with the entire set of polarizations rotated 90°. In fact, the same results were always

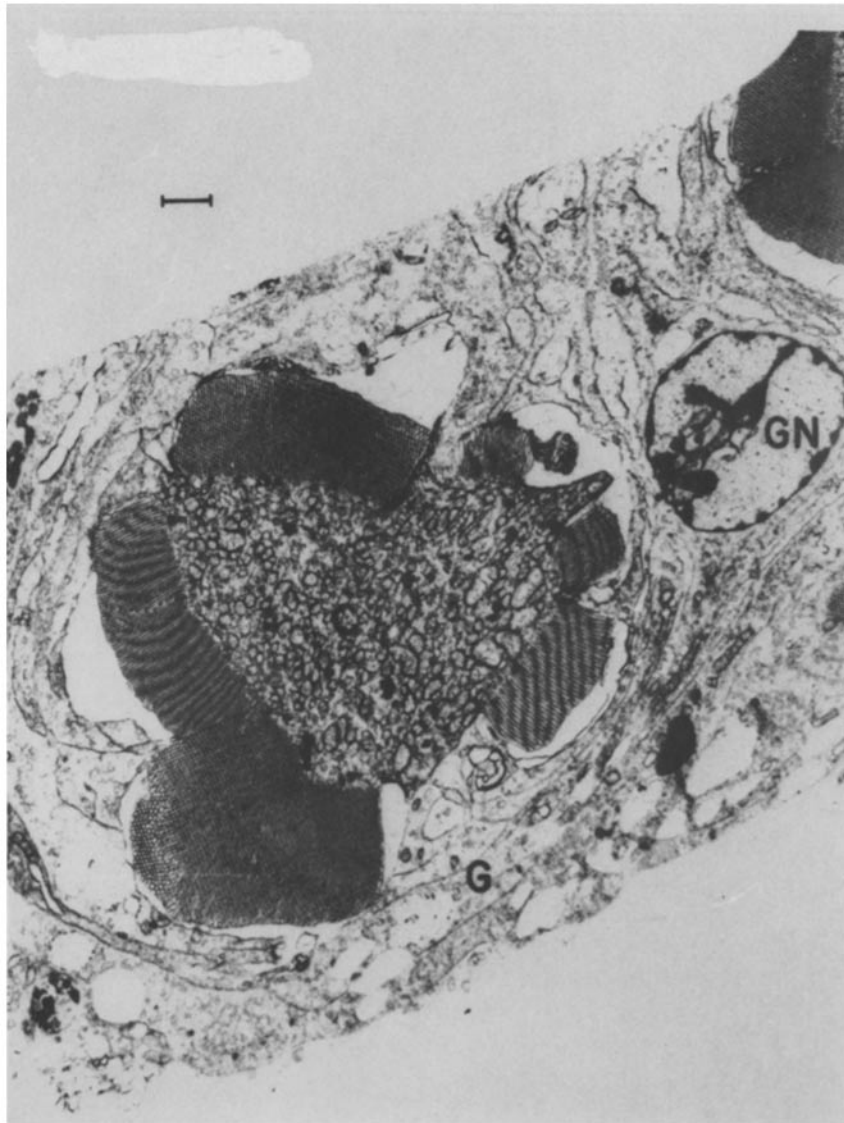


FIGURE 4. Rhabdomeric fine structure of the barnacle lateral ocellus photoreceptor. The cell projects multiple dendrites, mostly toward the tapetum. This is an electron microscope cross-section of one of these cell dendrites (C) with surrounding glial cell (G). The glial nucleus (GN) is also seen. The microvilli are outfoldings of the dendrite membrane, and are seen to be arranged in groups, each group containing many parallel microvilli with axes roughly radial to the dendrite axis. From many such sections, we calculate (see Appendix I) that, on the average, groups 6 μm apart have their microvillar axes perpendicular. The bar represents 1 μm .

found in both orientations, which confirmed that the populations were indeed roughly equal.

The results of one experiment are illustrated in Fig. 6. In each part, the responses to the same pair of stimuli after three different adaptations are shown superposed (*d* is a repeat of *b* to show stability). The stimuli are as depicted in Fig. 5: a blue anti-PDA-inducing stimulus (III) was followed by a polarized red PDA-inducing stimulus (IV). Trace sets *A* and *B* differ by 90° in the orientations of the polarizations, and their similarity confirms the equality of perpendicular

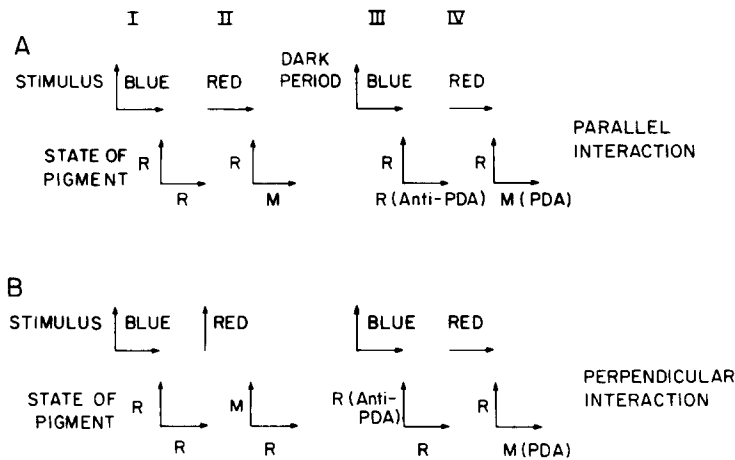


FIGURE 5. Scheme of an experiment to determine the range of the interaction of the PDA and the anti-PDA using polarized light. Polarized light is absorbed much more strongly in microvilli with axes parallel to its plane of polarization than perpendicular. Accordingly, a PDA and an anti-PDA induced by perpendicularly polarized stimuli will tend to be located in pigment populations separated by an average of 6 μm (Fig. 4), and their degree of interaction will be determined by their interaction range with respect to this distance. This figure sets out the succession of stimuli needed to induce the PDA and anti-PDA in separated pigment populations (*B*), and as a control, in coextensive populations (*A*). The upper row in each part shows the color and direction of polarization of the stimuli, all of which were saturating. The arrows indicate unpolarized light. The lower row indicates the predominant state of the pigment in the microvilli with axes along the two directions, after each stimulus, as well as the induction of a PDA or anti-PDA in each population.

populations as noted above. Traces *a* correspond to the “perpendicular” induction of PDA and anti-PDA and traces *c* correspond to the “parallel” induction. For traces *b* and *d*, step II of Fig. 5 was omitted, so that the blue stimulus was neutral and induced no anti-PDA.

The same amount of pigment was converted from R to M in all the traces of Fig. 6. However, when the PDA was induced in a pigment population coextensive with one in which an anti-PDA had been induced, PDA induction was strongly impeded (*c*). When the PDA and anti-PDA populations were separated, the PDA was larger (*a*). It was still smaller than in the unimpeded cases, *b* and *d*, as would be expected from the partial overlap between populations absorbing

perpendicularly polarized stimuli, caused both by the noninfinite value of the dichroic ratio and by the presence of intermediate angles of microvillar orientation.

If the range of the interaction between the PDA and anti-PDA were long compared with the average population separation, that separation would be irrelevant and traces *a* and *c* would coincide; that they do not sets an upper limit on the range, of the order of the average population separation.

Since the diffusion of the hypothetical transmitter depends on the range and on the time available, we wished to extend as much as possible the time between the anti-PDA induction and the PDA. This time is of course limited by decay of the anti-PDA.

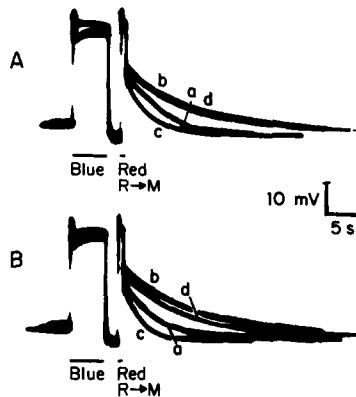


FIGURE 6. Is PDA induction in one population of microvilli fully impeded by prior anti-PDA induction in another, only partially overlapping, population, averaging $6 \mu\text{m}$ apart? Traces *c* and *a* in both parts (*A* and *B*) show the responses to stimuli III and IV of the upper and lower schemes of Fig. 5, respectively: coextensive and separated induction of PDA and anti-PDA. One sees that PDA induction is appreciably less impeded by the separated anti-PDA (*a*) than by the coextensive anti-PDA (*c*), which shows that the range of the interaction is not large compared with $6 \mu\text{m}$. Traces *b* and *d*, for comparison, show the PDAs resulting from the same red stimulus when no prior anti-PDA was induced; that is, the prior adaptation was blue, so that the blue stimuli were neutral. If the PDA and anti-PDA populations had been entirely separate and if there were no interaction spread and no light scatter, trace *a* would have coincided with *b* and *d*, but the population overlap was considerable. *A* and *B* differ in that the polarization directions of the whole set of stimuli were rotated 90° with respect to the cell (see text).

We followed the impeding effects of induction of anti-PDAs by parallel stimulations (Fig. 7, circles) and perpendicular stimulations (Fig. 7, triangles) through the time course of decay of the anti-PDA. This time course was measured by increasing the dark interval between steps III and IV of Fig. 5. The abscissa is the interval between the anti-PDA and PDA inductions, and the ordinate is the duration of the corresponding PDA to half-height. Both curves approached saturation at ~ 2 min, at which time the anti-PDA had almost fully died away. (The duration of the anti-PDA was also measured with diffuse light in this cell

and a similar result was obtained.) If significant diffusion of the PDA or anti-PDA processes had taken place during this period, we would have expected the two curves to converge on each other more quickly than they converged on the saturated level, since diffusion would cause both anti-PDA populations to approach a homogeneous distribution, and the difference between them would therefore disappear. In fact, no such tendency was seen. We chose for detailed analysis the 30-s point as the longest time at which the difference between the two curves was still strongly significant. In Appendix II, we calculate from the 30-s data of Fig. 7 an upper limit for the diffusion coefficient of a hypothetical transmitter. The fact that the difference between the two curves (Fig. 7) remained constant during the first 30 s suggests that there was no appreciable diffusion of the anti-PDA process within 30 s, and this is made quantitative in Appendix II.

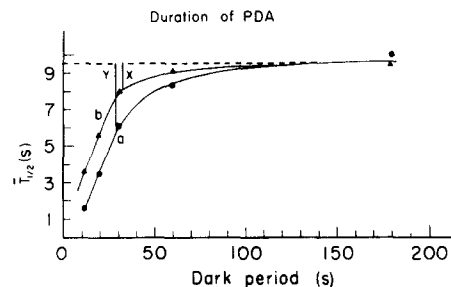


FIGURE 7. The time courses of the decay of the anti-PDA and the spread, if any, of the interaction between the PDA and the anti-PDA. If this spread were gradual, the interaction would increase with time, the relative difference between the effects on the PDA of the coextensive and separated anti-PDAs (Fig. 6) would decrease with the interval between the anti-PDA induction and the PDA induction, and the curves would converge on each other more rapidly than they do on their asymptote. The figure displays the half-height durations of the PDAs induced at various times after coextensive (circles) and separated (triangles) induction of anti-PDAs in an experiment similar to that of Fig. 6. Both curves are asymptotic to the unimpeded PDA duration as the anti-PDA decays, but they do not converge on each other more rapidly than on the asymptote, so no increasing spread of the anti-PDA interaction is indicated. The 30-s points (*X* and *Y*) were selected for more detailed analysis (see Appendix II).

DISCUSSION

The results of Figs. 1–3 show that the PDA process induced in one half of the cell was unaffected during its entire 30-s time course by the PDA depression and anti-PDA induced in the other half. This means that neither the PDA process nor the PDA depression or anti-PDA process diffused a substantial fraction of the cell diameter, or $\sim 50 \mu\text{m}$ in 30 s. By an indirect method, Figs. 4–7 reduce this limit in the anti-PDA to $\sim 6 \mu\text{m}$ in 30 s. These data lead to the following estimates of upper limits for the diffusion rates. Note that both interactions are known to take place quickly when the components are coextensive, and the measured time and space separations are therefore the relevant parameters for the diffusion calculation.

For the PDA depression experiment of Fig. 1, a diffusion constant of $D < 10^{-6}$ $\text{cm}^2 \text{s}^{-1}$ can be obtained if free homogeneous diffusion is assumed. This limit of course does not apply if (a) the diffusing element is the depressing agent and it has a lifetime shorter than 30 s, or (b) there is a barrier to free diffusion. Such a barrier might be either between microvilli or between blocks of microvilli (Fig. 4).

The same upper limit applies to the anti-PDA interaction from the half-cell experiments. This again is valid only for free diffusion, but the first reservation falls away, as the anti-PDA is known (Fig. 3B) to last longer (1 min) than the time interval involved.

The polarized light experiment lowers the upper limit on D for the anti-PDA interaction substantially, if indirectly. By making the analysis as quantitative as possible (see Appendix II), we obtain $D < 6 \times 10^{-9}$ $\text{cm}^2 \text{s}^{-1}$; since several of the components of the calculation are independent limits, a value close to this limit is unlikely.

The anti-PDA may act either on the PDA excitatory transmitter or on its source. Hochstein (1979) suggests, mainly on the basis of the apparent identity of the ionic specificity of the LRP and PDA conductances, that they have the same excitatory transmitter. However, the anti-PDA does not substantially depress the LRP (Hochstein et al., 1973), so its interaction is presumably with the source of the PDA. In the preceding article, we suggested that this source may comprise complexes of pigment molecules. If the source of the anti-PDA is within the same complexes, the low upper limit on the range and rate of diffusion of the interaction between the PDA and the anti-PDA is explained.

CONCLUSIONS

Previous work on the barnacle photoreceptor has shown that the interactions between the PDA and PDA depression and anti-PDA have nonzero ranges. By using various techniques of localized pigment conversions, we have now placed upper limits on these ranges. These limits, assuming free diffusion, translate into diffusion coefficients $D < 10^{-6}$ and $< 6 \times 10^{-9}$ $\text{cm}^2 \text{s}^{-1}$ for the PDA interaction with the PDA depression and the anti-PDA, respectively. The latter value is restrictive and suggests that the PDA and anti-PDA may be confined to the pigment molecules.

APPENDIX I

Calculation of the Average Separation of the Pigment Populations Absorbing Linearly Polarized Lights of Mutually Perpendicular Orientation

We shall calculate the average separation of mutually perpendicular microvilli in the barnacle lateral ocellus. This will be an upper limit on the average separation of the two pigment populations because: (a) the dichroic ratio of microvilli is not infinite; (b) microvilli at intermediate angles absorb both orientations of light; and (c) light scattering degrades the polarization.

Fahrenbach (1965) and Krebs and Schaten (1976) have studied the ultrastructure of the lateral photoreceptors of *B. eburneus*. They showed that the microvilli occupy dendritic foldings of the cell membrane, mainly on the tapetal side of the photoreceptor. The

dendrites are $\sim 30 \mu\text{m}$ long and have diameters of $5\text{--}7 \mu\text{m}$. There are many closely packed dendrites. They do not seem to have any preferred orientation in space. The microvilli appear along the dendrites in patches of up to a few microns in diameter. In each patch, the microvilli are parallel and their long axes are more or less radial to the main axis of the dendrite.

In the present study, we have looked at many sections like that shown in Fig. 4, to determine the spatial correlation of the microvillar orientations. Since there is no preferred direction for the dendritic processes, any section through the cell, in any direction, gives a good representation of the microvillar orientations. Thus, one can assume that the specific section studied is normal to the direction of propagation of the light stimuli. The projected angles of the microvilli on the section plane will therefore determine the relative efficiency of the microvillus in absorbing light of a specific polarization.

Accordingly, to estimate the average distance between the two pigment populations (absorbing two mutually perpendicular light polarizations), one has to measure the average distance over which the projected angle of the microvilli, in the section plane, changes by 90° .

We examined in detail four sections in which there were 40 pairs of neighboring microvillar patches. (Each pair was clearly connected through the plasma membrane.) In each pair, we measured the angle (in the plane of the section) between the directions of the two patches. The average angle was 50° , with a standard deviation of 34° . The average distance along the plasma membrane between the centers of the two patches was $3 \mu\text{m}$, with a standard deviation of $1.6 \mu\text{m}$. There was no correlation between the angle and the distance of the two patches. The scatterings around the average, both for the distances and for the angles, were symmetrical.

We also looked at second-order neighbors of the microvillar patches. The average angle between second-order neighbors was 90° (42° SD), and the average distance was $6 \mu\text{m}$ ($3 \mu\text{m}$ SD). The 90° average is made up of a 100° average angle for cross-sections perpendicular to the dendritic axis as in Fig. 4, and an 80° angle for diagonal and longitudinal sections.

Both the first- and second-order results are compatible with an average separation of mutually perpendicular microvilli of $6 \mu\text{m}$, and this is the value we adopt for the upper limit on the separation of the two pigment populations absorbing mutually perpendicularly polarized light.

APPENDIX II

Calculation of an Upper Limit for the Diffusion Coefficient for the Anti-PDA Interactions in the Polarized-Light Experiment

We take the distances from the points to the saturated value of the PDA duration (X and Y in Fig. 7) as measures of efficiency of the anti-PDA at that time. The ratio X/Y , the relative efficiency of the perpendicular anti-PDA, has a value of ~ 0.5 and an upper limit of 0.7 . This implies that at this time the ratio of the lowest to the highest anti-PDA concentration is < 0.7 . The argument for this is made explicit in Fig. 8, which shows schematically the concentration distributions of hypothetical anti-PDA and PDA substances among the microvilli, as induced by the polarized-light procedures. The abscissa is marked $\pi/2$ or $0\text{--}6 \mu\text{m}$, which is the average distance between perpendicular microvilli. Curve c is the distribution (approximately \cos^2) of a PDA induced by light polarized at angle 0 . Curve a is a parallel anti-PDA, which was induced some time earlier and then decayed and diffused. Curve b is for a "perpendicular" anti-PDA and is anti-symmetrical to a . The amount of PDA reduction is represented by the falling-striped area for the

parallel anti-PDA and by the rising-stripped area for the perpendicular anti-PDA. This representation assumes a linear interaction of the anti-PDA with the PDA. We know that this is roughly so from the work of Hochstein et al. (1973). The ratio of the rising-stripped area to the falling-stripped area represents the relative efficiency of the perpendicular anti-PDA represented by X/Y in Fig. 7. From the drawing, one can see that the ratio of anti-

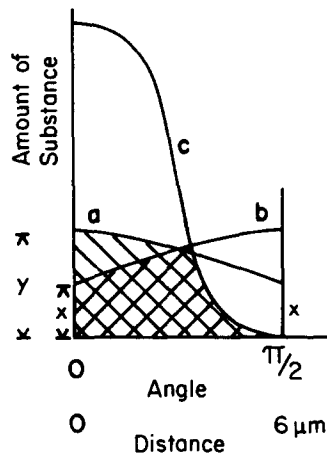


FIGURE 8. Demonstration that at some angle (0) the ratio of the anti-PDA concentrations from “perpendicular” and “parallel” stimulation at the time of PDA induction is smaller than the ratio of their effects on the PDA. The figure shows schematically the concentration of a hypothetical anti-PDA substance some time after its induction, during which time it has decayed and diffused, as a function of distance from the groups of microvilli with angle 0 . At this angle, anti-PDA production is maximal for “parallel” and minimal for “perpendicular” stimulation. The abscissa is marked $0-6 \mu\text{m}$, which is the average distance between perpendicular microvilli (Fig. 4 and Appendix I), and also the corresponding angle. Curve c is the PDA distribution. The curve is similar to a \cos^2 curve, which describes the cross-section for light absorption and would approximate the pigment distribution for low light intensities. Curves a and b represent the anti-PDA distribution for “parallel” and “perpendicular” stimulation, respectively. Their shape is what one expects to happen to curve c after a period of decay and diffusion. The amount of PDA reduction is represented by the rising-stripped area for the perpendicular anti-PDA and by the falling-stripped area for the parallel anti-PDA. From the drawing one can see that the ratio x/y of anti-PDA concentrations at angle 0 is necessarily smaller than the ratio of the areas, which corresponds to X/Y in Fig. 7. (This result holds for any diffusing substance and does not depend on the details of its initial distribution.) This puts a lower limit on the anti-PDA gradient after 30 min, and so an upper limit on its diffusion coefficient.

PDA concentrations at angle 0 is necessarily smaller than the ratio of the areas, which in turn is <0.7 . Because of the symmetry of the two anti-PDAs, this puts a lower limit on the anti-PDA gradient over a distance of $6 \mu\text{m}$ after 30 s and so an upper limit on its diffusion coefficient. That is, any diffusion of the anti-PDA “transmitter” has not reduced the concentration gradient so much that the ratio between the concentrations in the microvilli perpendicular and parallel to the stimulus polarization exceeded 0.7 .

To use this result to calculate an upper limit for the diffusion coefficient of the hypothetical transmitter, one must know the initial gradient. For ease of calculation, we assume a concentration homogeneous over a disk of radius $3 \mu\text{m}$ and zero elsewhere. The true distribution will certainly be less steep than this, so that the resulting diffusion constant D will remain an upper limit.

Carslow and Jaeger (1959) solved the disk problem, and, using their solution, we found that a value of 0.7 is obtained for the ratio of the concentration at a radius of $6 \mu\text{m}$ from the center of the disk to that at the center 30 s after time 0 if $D = 6 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$. This is therefore the upper limit on the diffusion constant for the interaction of the PDA and anti-PDA, assuming no diffusion barrier.

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