The Initiation of Excitation and Light Adaptation in *Limulus* Ventral Photoreceptors

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ABSTRACT Two types of experiments indicate that light adaptation and excitation are initiated by the same, rather than different, populations of visual pigment. (*a*) The criterion action spectra of light adaptation and excitation are the same. (*b*) Increment-threshold curves were measured with a voltage-clamp technique under conditions of high and low concentration of plasma membrane rhodopsin (Rh_{pm}). S_D, the dark-adapted sensitivity, and $1/I_2$, the inverse of the background irradiance that desensitized by 0.3 log units, underwent the same fractional change when the rhodopsin concentration was changed. Both quantities appear to be linearly related to Rh_{pm}. Reversible reductions in Rh_{pm} were achieved by orange irradiation during a brief increase of extracellular pH from 7.8 to 10. This procedure would be unlikely to produce similar concentration changes in a hypothetical intracellular pigment because the concurrent change in intracellular pH, measured using the dye, phenol red, was only 0.45 pH units. It is thus unlikely that an intracellular pigment initiates light adaptation.

On the assumption that light adaptation is mediated by a light-induced release of Ca^{++} from an intracellular store, the results reported here imply that an intracellular transmitter is needed to couple Rh_{pm} to the intracellular store.

INTRODUCTION

Like other photoreceptors, those of the *Limulus* ventral eye are both excited and desensitized by light. Excitation in these cells is a depolarization caused by an increase in sodium conductance (Millecchia and Mauro, 1969 *a*, *b*; Brown and Mote, 1974). Light adaptation (desensitization) is a process that reduces the amount of depolarization per absorbed photon. This desensitization is due primarily to a reduction in the conductance increase per absorbed photon (Lisman and Brown, 1975 *a*, *b*). How these processes are initiated by the absorption of light is unclear. One possibility is that a single population of visual pigment initiates both excitation and light adaptation. Alternatively, the cell may contain two visual pigments, one that initiates excitation, and a second that initiates light adaptation.

These models are of particular interest in connection with the role of calcium in light adaptation. Several lines of evidence indicate that a rise in intracellular

free calcium (Ca_i) mediates light adaptation in *Limulus* ventral photoreceptors (Lisman and Brown, 1972 *a*, *b*; Brown and Blinks, 1974; Lisman and Brown, 1975 *b*; Fein and Lisman, 1975; Brown and Lisman, 1975; Fein and Charlton, 1977 *a*). Direct measurement of Ca_i indicates that Ca_i goes up in the light (Brown and Blinks, 1974; Brown et al., 1977). Because this rise in Ca_i is not greatly reduced when extracellular Ca is removed (Lisman, 1976), it seems likely that light initiates the release of Ca^{++} from intracellular stores. Invertebrate photoreceptors contain intracellular cisternae in the light-sensitive parts of the cell, and recent experiments in honeybee indicate that the cisternae contain high concentrations of Ca^{++} (Perrelet and Baede, 1978). Similar structures in *Limulus* ventral photoreceptors are a likely site for intracellular Ca^{++} release, but there is no evidence bearing directly on this point.

Whatever the site of intracellular Ca^{++} release, the release must be initiated by the cell's visual pigment. The principal sites of invertebrate visual pigment are the microvilli which are infoldings of the plasma membrane. Thus, if visual pigment in the plasma membrane initiates Ca^{++} release from an intracellular compartment, some sort of message must be sent from the plasma membrane to the intracellular compartment. The alternative possibility is that the membrane of the intracellular compartment contains its own visual pigment which initiates the Ca^{++} release. Thus excitation might be mediated by visual pigment in the plasma membrane, whereas light adaptation might be initiated by an intracellular pigment. A dual pigment model is not implausible in view of the existence of two visual pigments in outer segments of squid photoreceptors (Hara and Hara, 1976) and in view of the recent evidence suggestive of two pigments in *Limulus* ventral eye.[1]

To determine whether excitation and light adaptation are initiated by the same or different populations of visual pigment, we have used two approaches. The first was to measure the criterion action spectra of excitation and light adaptation. If the two processes have different spectra, two pigments would be clearly implicated. The second approach was to alter selectively the concentration of visual pigment in the plasma membrane and then to determine whether excitation and adaptation were similarly affected, as predicted by the single-pigment hypothesis, or whether excitation was selectively affected, as predicted by the two-pigment hypothesis. A preliminary report of this work has been presented (Lisman and Strong, 1978).

MATERIALS AND METHODS

Limulus were obtained from the Marine Biological Laboratory, Woods Hole, Mass., and stored in artificial seawater for up to several weeks before use. The ventral nerve was dissected out under bright white light and mounted in a transparent perfusion chamber which was placed on the stage of a compound microscope (Fig. 1). The perfusion chamber had a glass roof (see Fig. 1, legend) which was particularly needed in experiments in which intracellular pH was measured by determining the cell's transmittance. The advantage of this chamber was that changes in flow rate, which occurred when the superfusate was changed, did not affect the water level above the cell and thus did not affect the optical path. During experiments, the nerve was superfused with

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[1] Lisman, J. E., P. K. Brown, H. Dubin, and R. Elliott. Manuscript in preparation.

artificial seawater (ASW) containing 450 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 26 mM $MgSO_4$, 22 mM $MgCl_2$, and 15 mM Tris, adjusted to pH 7.8. To make pH 10 superfusate, Tris was replaced with glycine, and, to minimize the formation of $Mg(OH)_2$ precipitate, $MgSO_4$ was replaced with Na_2SO_4 and the $MgCl_2$ concentration was reduced to 7.5 mM.

Cells were impaled with one or two microelectrodes filled with 3 M KCl (resistance of 12 to 25 MΩ). The voltage-clamp circuitry was as described in Lisman and Brown, 1971. In some experiments, an Ortec 4620 signal averager was used (Ortec Inc., EG&G, Inc., Oak Ridge, Tenn.).

A Strobonar 710 (Honeywell, Inc., Test Instruments Div., Denver, Colo.) was used to evoke the early receptor potential. Other illumination was provided by two tungsten iodide sources powered by regulated DC power supplies and modulated by electro-mechanical shutters. Light from these three sources was combined and focused onto the preparation through the microscope condenser (Fig. 1). The beams were made mono-chromatic with Corion Corp. (Holliston, Mass.) filters (half-bandwidth 10 nm) and attenuated with neutral density filters which were calibrated at each wavelength used. Light transmitted through the preparation was collected by a Vickers Instruments, Inc.
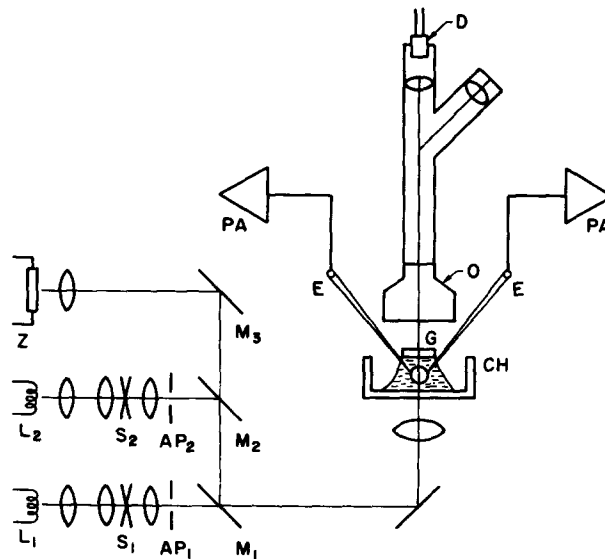

FIGURE 1. Apparatus for stimulating and recording from ventral photoreceptors. Light from a xenon flash (Z), and two quartz iodide bulbs (L_1 and L_2) is combined with a half-silvered mirror (M_1), a piece of glass (M_2), and a mirror (M_3). Light from L_1 and L_2 can be independently controlled by electromechanical shutters (S_1 and S_2). Light is focused onto the photoreceptor contained in a transparent chamber (CH). With apertures (AP_1 and AP_2), light can be restricted to a small region centered on the cell. The cell is impaled with two microelectrodes (E) which are attached to preamplifiers (PA). Above the cell is a long narrow piece of glass (G) whose ends rest on the side of the chamber (not shown). The superfusate is restricted to the region of the chamber below the glass because of attraction between the solution and the glass and repulsion between the solution and the silicone that covers the bottom of the chamber. Light transmitted through the cell is collected by the long working-distance objective (O). A trinocular microscope head allows simultaneous viewing of the preparation and measurement of trans-mitted light with the photodiode (D).

(Woburn, Mass.) 40X long working-distance reflecting objective and focused onto a PV-040 photodiode (Fig. 1; EG&G Inc., Salem, Mass.). Current from the photodiode was measured with a Burr-Brown 3523L operational amplifier (Burr-Brown Research Corp., Tuscon, Ariz.). This system was calibrated against a PIN 10 photodiode (United Detector Technology Inc., Santa Monica, Calif.).

To determine the relative irradiances of monochromatic lights incident on the cell, we removed the microelectrode (at the end of an experiment) and recorded photodiode outputs for the unattenuated beams at each wavelength. The diode was positioned to measure the light passing through a 50-μm Diam circle centered on the cell. This method actually measured the light transmitted through the cell, but because the cells are virtually colorless, the transmitted light should have yielded a reasonably accurate measure of the relative incident irradiances at different wavelengths.

To measure intracellular pH, we pressure-injected (3.4 × 10^4–13.8 × 10^4 N/m^2) a phenol red solution into the cell through a microelectrode. The injection solution contained 100 mM phenol red (Eastman Organic Chemicals Div., Rochester, N.Y.) and 200 mM Hepes, and was adjusted to pH 7 by the addition of KOH. The tip of the electrode had been broken until the resistance was approximately 20 MΩ in ASW. The photodiode was positioned to detect light transmitted through a 20 μm Diam region of the cell. Illumination was restricted to a 100 μm region centered on the cell by apertures in the light beams (AP$_1$ and AP$_2$ in Fig. 1) to reduce stray light.

Calibration curves (Fig. 2) for phenol red were made on a Cary spectrophotometer (Varian Associates, Palo Alto, Calif.) using a dye concentration of 1.4 × 10^{-5} M in a 1-cm cuvette. The resulting calibration curve is almost identical to one made by J. E. Brown and P. K. Brown (unpublished data) using higher dye concentration and a path length (100 μm) more comparable to the diameter of a ventral photoreceptor. To determine if the indicator properties of the dye were influenced by ionic strength or by protein, absorption spectra were recorded with and without 0.5 M KCl, and with and without 20 mg/ml bovine serum albumin. In each case the pH of the solution was adjusted to 7.2. The absorption spectra of the dye were the same in each case, indicating that neither ionic strength nor the presence of protein significantly affects the indicator properties of the dye.

RESULTS

Action Spectra of Excitation and Light Adaptation

It is of interest to compare the action spectra of light adaptation and excitation because differences in the spectra would indicate that these processes are initiated by different pigments. To find the action spectrum of excitation, we measured, at several wavelengths, the intensity of the monochromatic flash required to elicit a criterion depolarization. To find the action spectrum of light adaptation, we measured, at several wavelengths, the intensity of a steady adapting light needed to produce a criterion desensitization. (This strategy is similar to that used by Stiles, 1939; Fuortes et al., 1961; and Fain, 1976.)

The details of the procedure for determining the criterion action spectrum of excitation were as follows. Cells were adapted with a 530-nm background light. It was desirable to do these experiments in light-adapted cells because the background light reduced base-line noise, reduced variations in the response to a flash, and eliminated the regenerative responses sometimes seen in dark-adapted cells. Monochromatic, 10-ms flashes of varying intensities and wave-

lengths were superimposed on the plateau of the response to the constant background light. At each flash intensity and wavelength, five flashes were given at an interflash interval of 10 s and the responses were averaged. The average incremental response ($\overline{\Delta V}$) was approximately linearly proportional to flash intensity for $\Delta V < 10$ mV. The criterion value of ΔV was chosen to be in this range. Two flash intensities, differing by 0.15 log units, were given at each wavelength; these intensities were chosen to give responses that bracketed the selected criterion value of ΔV. The flash intensity I (λ) which would elicit the criterion response was then found by interpolating between the two measured points.
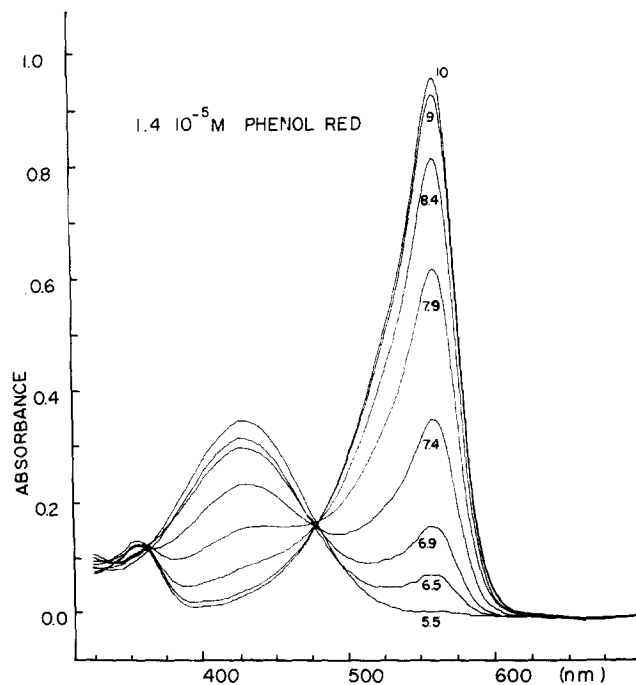

FIGURE 2.   Absorption spectra of phenol red solutions at different pHs measured on a Cary spectrophotometer. The path length was 1 cm.

Each I (λ) was compared with the average ($\overline{I\ (530)}$) of the values of I (530) measured immediately before and after I (λ) was measured. Two sequential values of I (530) usually agreed to within 2%. This method was used because preliminary experiments showed that, over the long periods of time needed to complete the experiment (~10 h), sensitivity sometimes changed by as much as 20%. The ratio I (530)/I (λ) was used for plotting the criterion action spectra of excitation.

The action spectrum of light adaptation was determined in an analogous manner. The cell was adapted with monochromatic lights of various intensities and wavelengths. Once the response to the adapting light had reached a stable plateau, several standard, 530-nm 10-ms flashes were superimposed on the

adapting light, and $\overline{\Delta V}$ was determined. In the intensity range of adapting light used, the response to the standard flash was approximately inversely proportional to the intensity of the adapting light. Two different intensities of adapting light were presented at each wavelength, and the adapting intensity which reduced the response of the standard flash to a criterion value was found by interpolating between the two measured points. As in the excitation section of the experiment, measurements at 530 nm were alternated with other wavelengths. Adapting lights used in both the adaptation and excitation sections of the experiment had intensities on the order of 5×10^{-6} W/cm^2 at 530 nm, and caused maintained depolarizations of 6-8 mV, with a plateau noise of 1-2 mV.
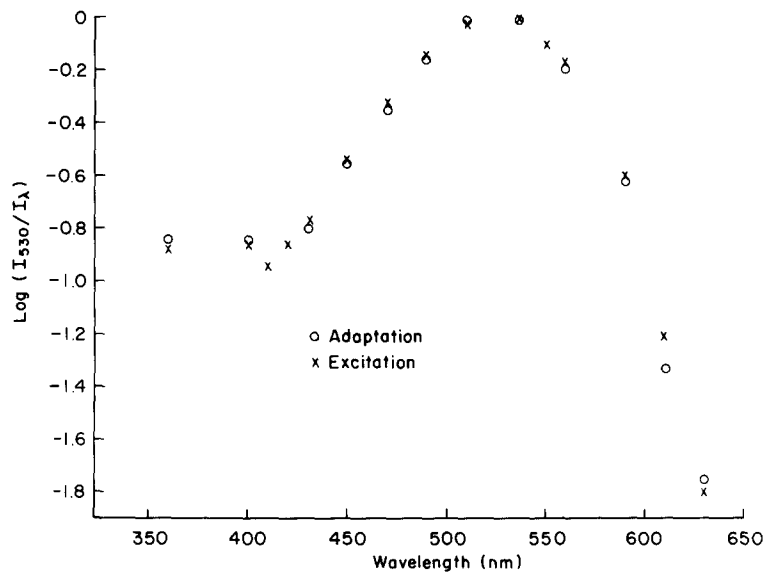

FIGURE 3. Criterion action spectra of excitation and light adaptation measured in the same cell. Both spectra are normalized relative to 530 nm. The technique for measuring the spectra is described in Results and Materials and Methods.

The background light that produced the criterion desensitization produced a plateau response that was independent of wavelength. Also, the waveform of the criterion amplitude response to the test flash did not depend on the wavelength of the adapting light or of the test flash. These observations indicate that the univariance principle was obeyed (Naka and Rushton, 1966).

The criterion action spectra of excitation and adaptation determined in the same cell are shown in Fig. 3 and appear to be identical. Similar determinations in three other cells revealed no significant differences between the two spectra. The peak of the spectrum was at 525 ± 5 nm, in agreement with previous measurements of the excitation spectrum (Millecchia et al., 1966; Nolte and Brown, 1970). The half-bandwidth was ≃100 nm, somewhat narrower than the cattle rhodopsin nomogram, in agreement with the results of Nolte and Brown (1970).

The close correspondence of the action spectra of light adaptation and excitation strongly suggests that the same pigment controls both processes. However, one could still postulate that two pigments with the same absorption spectrum, but different locations, control the two processes. In view of this limitation, we designed further experiments based on the entirely different strategy described in the section below.

Effects of Shifting Rhodopsin Concentration

According to the two-pigment hypothesis described in the Introduction, plasma-membrane rhodopsin initiates excitation, whereas an intracellular pigment initiates light adaptation. A strategy for testing this hypothesis is to reduce selectively the concentration of plasma-membrane rhodopsin. The two-pigment hypothesis predicts that excitation and adaptation should be differentially affected. The single-pigment hypothesis predicts a parallel alteration of the two functions. In section A we describe how light and extracellular pH can be manipulated to establish a low or high concentration of plasma-membrane rhodopsin. In section B we define quantitative measures of excitation and light-adaptation and describe the results obtained in the low and high rhodopsin states. In section C we show that intracellular pH (pH_i) is relatively insensitive to changes in extracellular pH (pH_o). This observation, together with the fact that the pH-sensitive site on the plasma-membrane pigment is exposed to the extracellular space, provides the basis for arguing that the plasma-membrane pigment is selectively affected by the pigment-shifting procedures.

A. PROCEDURE FOR ALTERING RHODOPSIN CONCENTRATION Studies of the early receptor potential (ERP) in *Limulus* ventral photoreceptors (Lisman and Sheline, 1976) indicate that *Limulus* rhodopsin is very similar to squid rhodopsin (Hubbard and St. George, 1958; Hagins and McGaughy, 1967). Light converts *Limulus* rhodopsin to a stable photoproduct, metarhodopsin. When metarhodopsin absorbs a photon it is converted back to rhodopsin. There is no dark-regeneration of rhodopsin on the time scale of our experiments. Metarhodopsin, but not rhodopsin, has pH indicator properties. At physiological pH (7.8), metarhodopsin exists in the "acid" form which has a λ_{max} almost identical to that of rhodopsin (525 nm). At high pH metarhodopsin exists in the "basic" form, and the λ_{max} shifts to the ultraviolet. The pK of this titration is approximately 8.5.[2]

The procedure for shifting pigment concentrations was as follows. At the start of the experiment, rhodopsin and acid metarhodopsin were in a photoequilibrium established by the bright lights used during the dissection. pH_o was raised to 10 for 3 min. During this time the cell was exposed to a 530-nm stimulus. This long wavelength irradiation converted most of the pigment to alkaline metarhodopsin. When the pH was then lowered to 7.8 in the dark, most of the pigment was in the acid metarhodopsin form. This we call the low rhodopsin state. After measurement of the excitation and adaptation properties in the low rhodopsin state, the cell was exhaustively irradiated with long wavelength light (either 530 nm or long pass filter) at pH 7.8. This reestablished

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[2] Lisman, J. E., and S. R. Schulman. Unpublished data.

a photoequilibrium of rhodopsin and metarhodopsin, which we call the high rhodopsin state. The cycle can be repeated.

The ERPs recorded during such a cycle are shown in Fig. 4. The ERPs were evoked by flashes filtered to transmit wavelengths longer than 475 nm. Both rhodopsin and acid metarhodopsin absorb light from such flashes, but alkaline metarhodopsin does not. The ERP waveforms have two components: absorption of light by rhodopsin generates a monophasic-negative wave, and absorption of light by acid metarhodopsin generates a faster rising monophasic-positive wave. In Fig. 4 a the pH was 7.8, rhodopsin and metarhodopsin were in photoequilibrium, and the ERP was biphasic. After the pH was raised to 10, most of the acid metarhodopsin was converted to basic metarhodopsin, and the flash evoked a large monophasic negative wave (Fig. 4 b) due mainly to rhodopsin. After
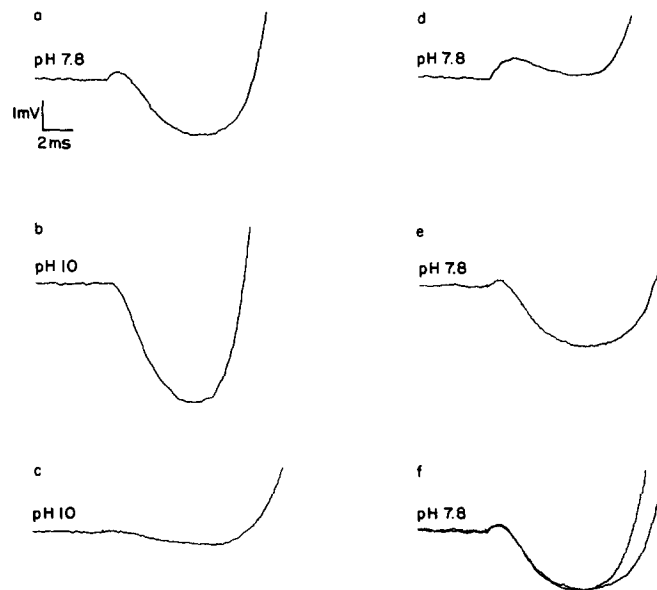
FIGURE 4. Early receptor potentials (ERP) at different stages of the pigment cycle. The cycle was generated by manipulation of light and extracellular pH. All ERPs were evoked by saturating 1-ms flashes filtered to transmit wavelengths longer than 475 nm. (a) ERP generated by a photoequilibrium mixture of rhodopsin and acid metarhodopsin. (b) ERP evoked by first flash 100 s after raising pH from 7.8 to 10.0. Primarily rhodopsin contributes to this ERP. (c) ERP evoked after 90 s 530 nm irradiation at pH 10. The small ERP is generated by a residual amount of rhodopsin in photoequilibrium with a small amount of acid metarhodopsin. (d) The ERP evoked 3 h after lowering pH to 7.8 This ERP is generated primarily by acid metarhodopsin. (e) ERP generated by photoequilibrium mixture of rhodopsin and acid metarhodopsin induced by exhaustive long wavelength irradiation. (f) Comparison of the ERP at the beginning (a) and end (e) of pigment cycle shows no significant loss of amplitude. The fast rising part of each waveform that begins approximately 200 ms after the onset of the response is the late receptor potential (LRP). LRP latency is very sensitive to the state of dark adaptation (Brown and Lisman, 1975). This probably accounts for difference in latency between a and e.

exhaustive 530-nm irradiation, which converted most of the rhodopsin to basic metarhodopsin, the ERP was greatly reduced in amplitude (Fig. 4 c). When the cell was brought back to pH 7.8, most of the pigment was in the acid metarhodopsin state, and the ERP was monophasic positive (Fig. 4 d). After exhaustive illumination, a photoequilibrium mixture of rhodopsin and acid metarhodopsin was reestablished and the ERP was again biphasic (Fig. 4 e).

Treatment of the cell with pH 10 superfusate for short periods of time did not damage the cell to any significant extent. A comparison of the biphasic ERP at the beginning and end of a single cycle shows no significant reduction in amplitude (Fig. 4 f). To examine more systematically the possible damaging effects of pH 10 solution on rhodopsin, we repeated the above cycle 12 times. It was found that each cycle reduced the amplitude of the biphasic ERP at 7.8 by 3%, on the average. This suggests that exposure to pH 10 caused slow destruction of visual pigment (and/or a slow disorientation of the pigment in the membrane). We therefore attempted to keep exposures to pH 10 solution as short as possible. In one cell, we measured the capacitance before and after reducing the rhodopsin concentration. We detected no change in capacitance and assume that exposure to pH 10 superfusate produces negligible change in membrane area. The results in section B show that the effects on excitation and adaptation produced by one pigment-shifting cycle are completely reversible within the accuracy of our measurements.

The relative amount of rhodopsin in the low and high rhodopsin states can be readily estimated. The high rhodopsin state is a photoequilibrium mixture of rhodopsin and acid metarhodopsin. The photosensitivities of rhodopsin and acid metarhodopsin are approximately equal (Lisman and Bering, 1977). This implies that when rhodopsin and acid metarhodopsin are in photoequilibrium their concentrations will be nearly equal (i.e., 50% of the pigment is rhodopsin). The relative amount of rhodopsin in the low rhodopsin state can be calculated if one assumes that the residual ERP at pH 10 reflects incomplete conversion of acid metarhodopsin to alkaline metarhodopsin. The residual acid metarhodopsin must exist in photoequilibrium with rhodopsin. Inasmuch as both the large biphasic ERP at pH 7.8 and the residual ERP at pH 10 reflect photoequilibrium mixtures of rhodopsin and acid metarhodopsin, the fractional reduction in rhodopsin concentration can be calculated by taking the ratio of the two ERP amplitudes. This calculation assumes that the ERP waveform and amplitude generated by each pigment molecule are independent of pH, an assumption that is valid only to a first approximation (Lisman and Sheline, 1976). For the cell shown in Fig. 4, the fractional reduction in rhodopsin concentration was 0.63–0.73 log units (i.e., a factor of 4.3–5.4). It is necessary to give a range of values because of the relatively large error in measuring the amplitude of the residual ERP.

The log of the fractional reduction in rhodopsin concentration for several other cells is listed in Table I. The variation from cell to cell probably arose because of small differences in extracellular pH due to incomplete removal of the pH 7.8 solution. These differences could have been eliminated by longer exposure to pH 10 solution, but this course was undesirable because of the accompanying destruction of rhodopsin.

B. MEASUREMENT OF EXCITATION AND ADAPTATION IN LOW AND HIGH RHODOPSIN STATES   Cells were impaled with two KCl microelectrodes, one for measuring voltage and one for passing current under voltage clamp. To the extent that a component of light adaptation might occur as a secondary consequence of voltage changes, for instance through the action of voltage-dependent channels (see Pepose and Lisman, 1978), light adaptation would necessarily be influenced by changes in excitation properties. It was, therefore, important to use a voltage-clamp technique to eliminate this component of light adaptation.

To examine excitation and light adaptation, we needed to assay the cell's sensitivity under a variety of conditions. We measured sensitivity by determining the reciprocal of the intensity of a brief (10-ms) flash required to elicit a criterion (10-nA) current response. The criterion response was chosen to be in the range where light-induced current is linearly related to flash intensity (Lisman and

TABLE I

CELL PROPERTIES IN HIGH* AND LOW RHODOPSIN STATES

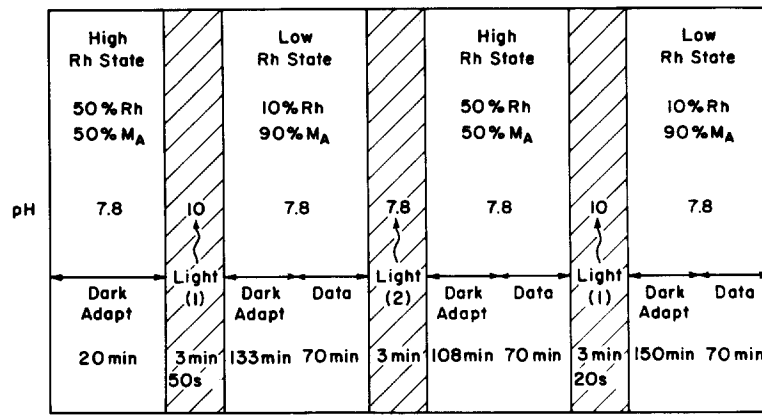| Cell no.	n	log ERP* − log ERP	log $1/S_D$ − log $1/S_D$*	log I_2 − log I_2*	Δlog $1/S_D$ − Δlog I_2
1	0.83	0.65‡ (0.60–0.70)	0.76	0.68	0.08
2	0.77	0.55 (0.50–0.61)	0.60	0.64	−0.04
3	0.68	0.88 (0.82–0.96)	0.96	0.97	−0.01
4	0.65	0.88 (0.77–1.0)	0.74	0.70	0.04
Average	0.73				0.02

* Signifies measurements made in high rhodopsin state.

‡ In the second trial of this experiment (see Fig. 5) the change in log ERP was 0.69 (0.64–0.75).

Brown, 1975 a). As in the previous experiments, the intensity required to elicit the criterion response was found by interpolating between the measured responses to two flash intensities which differed by 0.15 log units. When the cell's sensitivity was near its maximum values, there was great variability in the response to flashes, and we found it necessary to signal-average four to six responses at each flash intensity. An interflash interval of 30 s was used, because preliminary experiments showed that shorter interflash intervals sometimes led to a progressive decline in response amplitude. When the cell's sensitivity was not near-maximum, it was possible to use shorter interflash intervals, and to include fewer responses in each average.

After the pigment had been shifted to the low rhodopsin state, as described above, the cells were dark-adapted (see Fig. 5 for experimental protocol). Sensitivity was periodically measured, and the cell was considered completely dark-adapted when sensitivity had leveled off to a maximum value. The time-course of the dark adaptation in one cell is shown in Fig. 6. The figure shows that sensitivity increased over the time-course of an hour. This is somewhat slower than described by Fein and DeVoe (1973).

The dark-adapted (maximum) sensitivity measured in this way is a measure

| High Rh State 50%Rh 50%M$_A$ pH 7.8 Dark Adapt 20 min | 10 Light (1) 3min 50s | Low Rh State 10%Rh 90%M$_A$ 7.8 Dark Adapt 133min Data 70 min | Light (2) 3 min | High Rh State 50%Rh 50%M$_A$ 7.8 Dark Adapt 108min Data 70 min | 10 Light (1) 3 min 20s | Low Rh State 10%Rh 90%M$_A$ 7.8 Dark Adapt 150min Data 70 min |

Total ∿10.5 h

FIGURE 5. Experimental protocol. During the initial 20 min of the experiment, the cell was partially dark-adapted to determine if it was healthy enough to generate discrete waves. The hatched regions are periods where the pigment concentration was changed. Periods marked "data" indicate the time required to generate an increment-threshold curve. Light 1 was a 90-s exposure to a 3×10^{-4} W/cm² 530-nm stimulus. Light 2 in this experiment was five flashes (>475 nm) each of which was bright enough to saturate the ERP. In one of the other experiments, light 2 also consisted of exposure to 530 nm light equal to that of light 1.
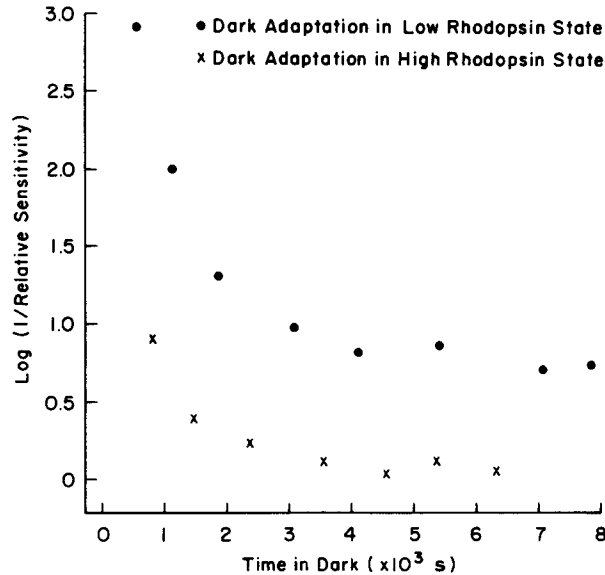
FIGURE 6. The time-course of dark adaptation in the low and high rhodopsin states. Zero time is taken as the end of the illumination used to generate the high rhodopsin state, and as the end of the exposure to pH 10 solution used to generate the low rhodopsin state. The dark-adaptation measurements in the low rhodopsin state were made during the initial trial (see Fig. 5) and were not repeated during the final trial.

of the excitation properties of the cell in the low rhodopsin state. To measure properties of light adaptation, we constructed an increment-threshold curve. The cell was light-adapted with steady lights of increasing intensity. After the response to the adapting light reached a stable value (60–90 s), sensitivity was measured. The increment-threshold curve is shown in Fig. 7 (same cell as Fig. 6). The background lights used were limited to a relatively small intensity range because bright lights would have altered the rhodopsin and metarhodopsin concentrations. After generation of the increment-threshold curve, the ERP was monophasic positive (Fig. 4 d). This indicates that the adapting lights converted relatively little of the acid metarhodopsin to rhodopsin, and that there was no
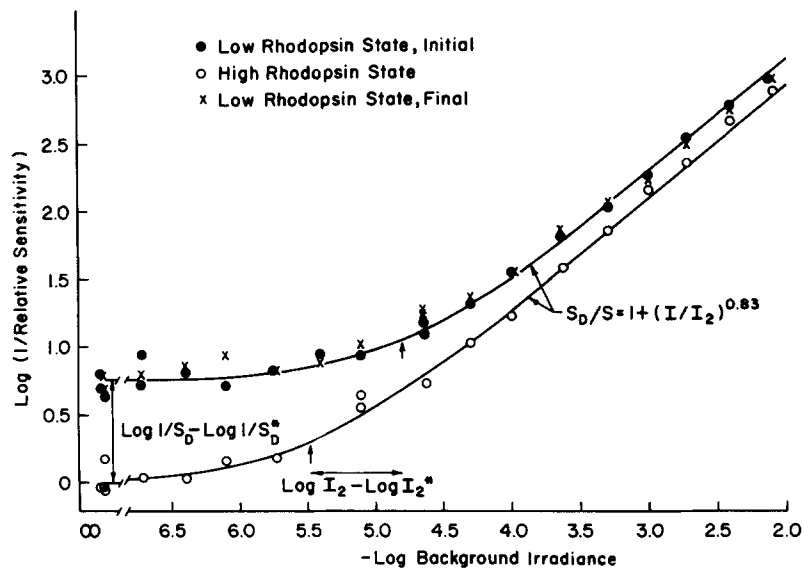

FIGURE 7. Increment-threshold curve in low and high rhodopsin states. The points furthest to the left represent the dark-adapted sensitivity in the absence of a background light. Irradiance of the 530-nm unattenuated beam (−log background irradiance = 0) was 3×10^{-3} W/cm^2. The solid lines are the template curve $S_D/S = 1 + (I/I_2)^{0.83}$ fit by eye to the data points. S is the sensitivity, S_D the dark-adapted sensitivity, I the background irradiance, and I_2 the irradiance that desensitizes by 0.3 log units (marked with vertical arrows). Starred quantities were measured in the high rhodopsin state.

spontaneous regeneration of rhodopsin. In passing it should be mentioned that results of this sort indicate that a large component of dark adapatation occurs (Fig. 6) without regeneration of rhodopsin, a conclusion consistent with that of Fein and DeVoe (1973).

Once excitation and adaptation had been assayed in the low rhodopsin state, the pigment was converted to the high rhodopsin state. The cell was again dark-adapted (Fig. 6) and the increment-threshold curve was constructed (Fig. 7). The maximum dark sensitivity reached a considerably higher value in the high rhodopsin state than in the low. For the cell shown in Figs 5, 6, and 7, the sensitivity increased 0.76 log units. Thus, when rhodopsin concentration was

raised, less light was required to excite the cell. Inspection of Fig. 7 reveals that less light was also required to desensitize the cell by any given amount. After the completion of measurements in the high rhodopsin state, the cell was returned to the low rhodopsin state. After dark adaptation, the absolute sensitivity and increment-threshold curve were again measured. The data (Fig. 7) superpose nicely with the initial low rhodopsin data made 6.7 h earlier.

The increment-threshold curves in Fig. 7 can be described by equations of the form

$$\frac{S_D}{S} = 1 + \left(\frac{I}{I_2}\right)^n,$$

where S_D is the dark-adapted value of the sensitivity (S) and I_2 is the irradiance of the background (I) that desensitizes by 0.3 log units. Because at high irradiances the equation reduces to $S_D/S = (I/I_2)^n$, n can be determined from the slope of the increment-threshold curve plotted on log-log coordinates. The exponent n varied somewhat from cell to cell (Table I), but in any one cell, n had the same value in the low and high rhodopsin states. Once n was determined, a template curve was generated and fit to the data by eye. S_D specifies the vertical location of the curve; I_2 specifies the lateral position of the curve.

The effects of changing rhodopsin concentration on S_D and I_2 are shown in Table I for four cells. Experiments of this sort were attempted on approximately 150 cells. Of these, most were discarded within 30 min after impalement because of their low sensitivity. Of the remaining 32 cells, 28 did not meet our long-term stability standards. Specifically, we required that cells not exhibit drifts in membrane potential > 10 mV, that they not exhibit spontaneous declines in sensitivity, and that neither microelectrode come out of the cell. In most of the 28 cells, one of the microelectrodes came out of the cell after 3 or 4 h, a time insufficient for study of both the low and high rhodopsin states. One of the four cells (cell 1) met our stability standards for over 10 h. Only in this cell was it possible to study the low rhodopsin state, then the high rhodopsin state, and finally return to the low rhodopsin state (see Fig. 5). It was thus possible in this cell to directly confirm the reversibility of all changes. All the results shown in Figs. 4–10 are from this experiment. In the three other cells, it was not possible to examine reversibility directly. In all experiments we examined first the low rhodopsin state and then the high. If the reverse sequence had been used, the lowered sensitivity in the low rhodopsin state might have been attributed to deterioration of the preparation.

The principal conclusion to be drawn from Table I is that, for any given cell, the change in the excitation parameter, $\Delta \log 1/S_D$, is approximately equal in magnitude to the change in the adaptation parameter, $\Delta \log I_2$. The largest difference is 0.08 log units which is within the error of these measurements. The correlation coefficient between I_2 and $1/S_D$ is 0.96. When the fractional changes in I_2 and $1/S_D$ are plotted against each other on a linear scale, the points can be fit by a linear regression line of slope 1.02. The equality of the shifts in excitation and adaptation parameters can be visualized without resort to the template curves. In Fig. 8 a the increment-threshold curve in the high rhodopsin
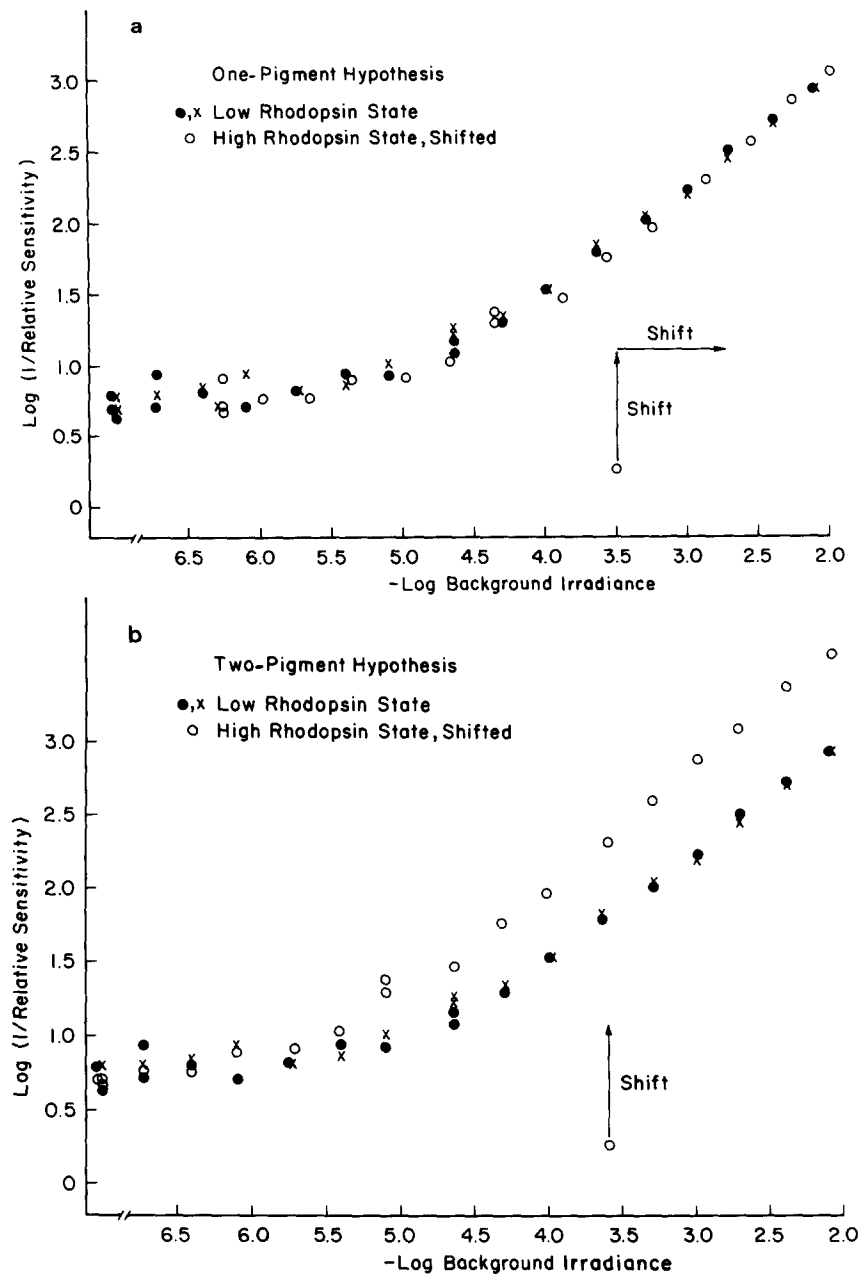
FIGURE 8. Tests of the one- and two-pigment hypotheses. (a) The increment-threshold curve in the high rhodopsin state has been shifted upward and to the right by 0.76 log units. The shifted points superpose with the low rhodopsin increment-threshold curve, in accordance with the prediction of the single-pigment hypothesis (see test). (b) The increment-threshold curve in the high rhodopsin state has been shifted upward by 0.76 log units, but has not been shifted laterally. As described in the text, such a shift is predicted by a plausible two-pigment hypothesis. The lack of superposition between the shifted data and the low rhodopsin increment-threshold curve argues against this two-pigment hypothesis.

state from Fig. 7 has been shifted upward and to the right by the same amount, $\Delta \log 1/S_D$ (0.76 log units). The fact that the shifted points superpose nicely with the low rhodopsin increment-threshold curve indicates that changing the rhodopsin concentration produces equal changes in the excitation and adaptation properties. Equal vertical and horizontal shifts would be expected if excitation and light adaptation are controlled by the same pigment, or by two different pigments whose concentration changes are closely correlated.

In Fig. 8 *b* the increment-threshold curves from Fig. 7 are plotted with the high rhodopsin curve shifted up by $\Delta \log 1/S_D$. The lack of overlap clearly shows that a vertical shift alone cannot account for the changes in the increment-threshold curve. A purely vertical shift would be expected if excitation were controlled by plasma-membrane rhodopsin and light adaptation were controlled by an intracellular pigment which had the same concentration in the low and high plasma-membrane rhodopsin states. An intracellular pigment would be likely to have approximately the same concentration in both states because it would be subject to the same irradiation (Fig. 5) and nearly the same intracellular pH (see section C) during the procedure for lowering or raising the concentration of plasma-membrane rhodopsin. Spectrophotometric measurements of what is thought to be an intracellular pigment in the ventral eye indicate that its concentration is nearly the same 2 h after shifting plasma-membrane rhodopsin to either a low or high concentration (see footnote 1).

A further conclusion to be drawn from Table I is that the fractional changes in excitation and adaptation parameters are approximately equal to the fractional change in rhodopsin concentration as measured by the ERP. When S_D*/S_D and I_2/I_2* are plotted against ERP*/ERP, the linear regression lines have slopes of 1.04 and 0.98, respectively. Given the inaccuracy of our measure of rhodopsin concentration and given the small range of the concentration changes, the conclusion that S_D and $1/I_2$ are linearly related to rhodopsin concentration should be considered tentative.

Figs. 9 and 10 illustrate two additional effects of changing the rhodopsin concentration, neither of which has been studied in detail. Fig. 9 shows the discrete waves evoked by a dim steady stimulus in both high and low rhodopsin states. It is apparent that the principal effect of lowering the rhodopsin concentration is to reduce the probability that an incident photon evokes a discrete wave. In contrast, background lights desensitize the cell primarily by reducing the size of discrete waves while leaving the probability of evoking them nearly constant (Dodge et al., 1968). Fig. 10 shows the responses to flashes superposed on background lights. For a given background intensity, the time-to-peak of the average response to a brief flash is longer when the cell is in the low rhodopsin state than the high rhodopsin state. However, if responses in the low and high rhodopsin states are compared, not for identical adapting lights, but for two adapting lights which caused approximately the same relative decrease of sensitivity in the low and high rhodopsin states, then the times-to-peak are the same. This is consistent with the notion that the shortening of the time scale caused by an adapting light (Fuortes and Hodgkin, 1964) is also initiated by plasma-membrane rhodopsin.

c. THE DEPENDENCE OF pH_i ON pH_o The question examined in this section is whether the pigment-shifting procedure described in section A would be

expected to produce similar changes in concentrations of the plasma-membrane pigment and an intracellular pigment. Because the pH-sensitive site on plasma-membrane metarhodopsin is exposed to the extracellular space (Lisman et al., 1975), this site responds directly to the changes in extracellular pH that are part of the pigment-shifting procedure. If intracellular pH (pH_i) closely followed extracellular pH (pH_o), then both the intracellular pigment and the plasma-membrane pigment might undergo similar concentration changes. Alternatively, if pH_i is relatively insensitive to pH_o, then the two pigments would be subject to quite different conditions, and similar concentration changes would be unlikely.
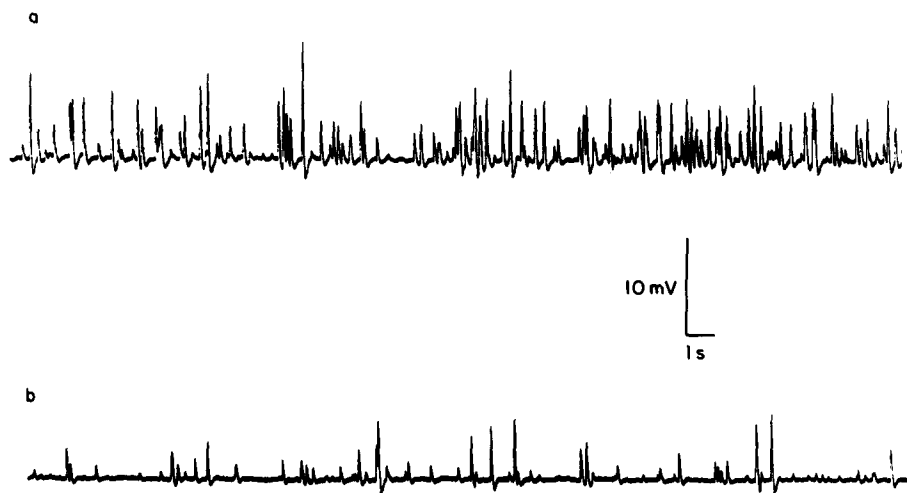
a

10 mV

1 s

b

FIGURE 9. The effect of changing the rhodopsin concentration on the light-induced discrete waves. A steady dim 530 nm stimulates the cell in the high rhodopsin state (a) and the low rhodopsin state (b). The main effect of changing the rhodopsin concentration is to lower the rate of discrete waves. There may be a small effect on wave amplitude, but the data are not sufficient to establish this.

Previous work on the ventral photoreceptor (Lisman et al., 1975) showed that pH_i is relatively insensitive to pH_o; however, the experiments were done under conditions quite different from the electrophysiological experiments reported here. It therefore seemed important to measure changes in pH_i under conditions identical to those used for the electrophysiological measurements. Fig. 11 shows the optical transmission of a ventral photoreceptor, mounted in a perfusion chamber (see Methods), as a function of time and pH_o. The cell was alternately exposed to flashes of 430 and 560 nm. The former generated a somewhat larger output of the photodiode. At the two vertical arrows, the pH indicator dye, phenol red, was injected into the cytoplasm of the cell by pressure injection through an intracellular microelectrode. This electrode was also used to record membrane potential (bottom trace, Fig. 11). Diffusion of the dye within the cell required several minutes. pH_i could be calculated by computing the absorbance at the two wavelengths, taking the ratio, and determining from calibration curves the pH that corresponded to this ratio. Fig. 12 shows the

results from five cells. At physiological pH (7.8), the average pH_i was 7.04 ± 0.03 (SE). This is in excellent agreement with the value of 7.01 ± 0.04 (SE) determined with intracellular pH electrodes in the ventral photoreceptor by Levy and Coles (1977).

When pH_o was raised to pH 10, transmission at 430 nm rose and transmission at 560 nm declined. When pH_o was returned to 7.8, the transmission changes were slowly reversed. From these transmission changes, the absorbances of the preparation at the two wavelengths could be computed as a function of time. The changes recorded, however, were due in part to absorbance changes of the preparation, as shown in the right section of Fig. 11. Here we have recorded the transmission changes of a nearby cell not injected with dye. The absorbance change of the dye itself was computed by subtracting the absorbance changes
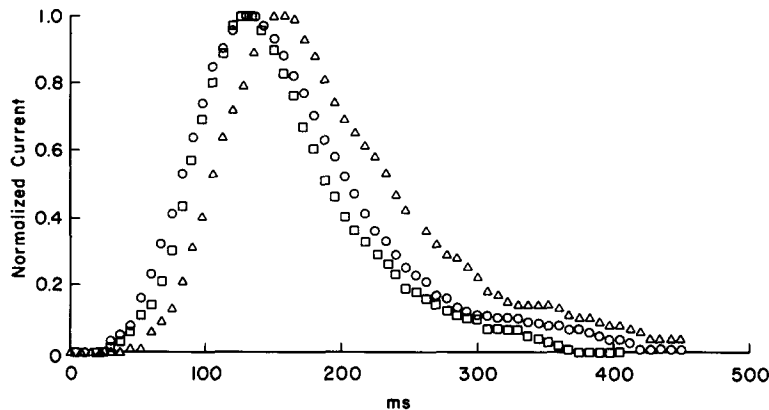

FIGURE 10.   Normalized incremental responses to brief test flashes in the low and high rhodopsin states. (□) High rhodopsin state, − log background intensity = 3.3. (O) Low rhodopsin state, − log background = 2.3. This background produced nearly the same desensitization as 3.3 background in high rhodopsin state. (△) Low rhodopsin state, − log background intensity = 3.3.

measured in the uninjected cell from the absorbance changes recorded in the injected cell. This correction was relatively small. From these corrected absorbances, the absorbance ratio was computed and the corresponding pH was determined from calibration curves. Fig. 12 shows that pH_i did rise when pH_o was raised from 7.8 to 10, but in a 3-min period this rise was, on the average, only 0.42 pH units. This is only about 2% of the change in extracellular proton concentration. We conclude that pH_i is quite insensitive to pH_o under the conditions used for shifting the plasma-membrane rhodopsin concentration. The insensitivity of pH_i to changes in pH_o is in agreement with results in other neurons (Bicher and Ohki, 1972; Thomas, 1974).

Using the molar extinction of phenol red at pH 7, and assuming a cell diameter of 50 μm, we estimate that the concentration of phenol red was <5 mM. Because pH indicator dye is itself a pH buffer and is injected together with pH buffer, Hepes (see Methods), it might be argued that the change in pH_o would be much larger in the absence of these buffers. Two related arguments

make this very unlikely. First, the added buffer is unlikely to have much effect on the buffering capacity of the cell if the intrinsic buffering capacity is as large (the equivalent of 150 mM pH buffer) as that in other cells (Thomas, 1974). Second, in two experiments (Lisman, J. E. and P. K. Brown, unpublished data) *Limulus* ventral photoreceptors were injected with dye buffered to pH 8 with an equimolar concentration of Tris. Absorbance measurements of the injected cell made in the Harvard University microspectrophotometer yielded a pH of 6.9,
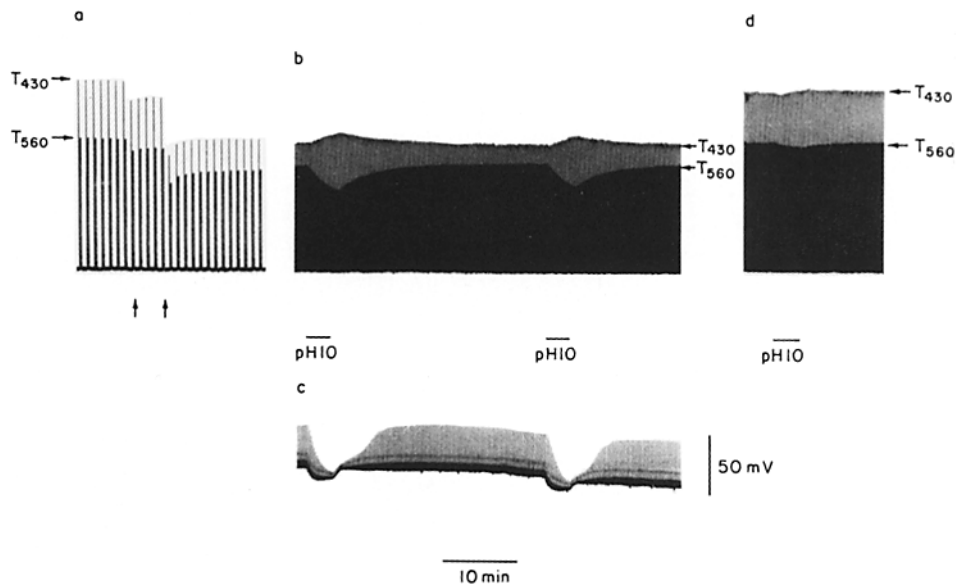

FIGURE 11.   The effect of raising extracellular pH on the transmission (T) of a single ventral photoreceptor injected intracellularly with the pH indicator dye, phenol red. Transmission was measured at the two peaks of the dye absorption spectrum (430 and 560 nm) by giving pairs of flashes, the first at 430 nm and the second at 560 nm. Pairs of flashes were given once every 10 s. Light transmitted through the cell was collected by a microscope condenser (see Fig. 1) and focused onto a photodiode. The photodiode output was measured with a current-to-voltage transducer that gave an upward voltage deflection proportional to the light intensity. T is thus proportional to the amplitude of the deflection. (*a*) T (560) and T (430) before and after dye injection which occurs in two spurts marked by the vertical arrows. pH$_o$ was 7.8. (*b*) T(560) and T(430) during two sequential exposures (approximately 3 min duration) to pH 10 superfusate. (*c*) Membrane potential monitored simultaneously with transmission changes in *b*. Resting potential in this cell is not known because electrodes containing high concentration of phenol red do not accurately measure DC voltages. (*d*) The transmission changes during exposure to pH 10 superfusates in a nearby cell not injected with dye.

indicating that the buffering capacity of the cell was much larger than that of the injected dye.

DISCUSSION

The experiments reported in this paper support the conclusion that plasma-membrane rhodopsin initiates both excitation and light adaptation. The critical

evidence is that changing the concentration of plasma-membrane rhodopsin produces parallel changes in the amount of light needed both for excitation and for any given desensitization (Fig. 7; Table I). The counter-hypothesis is that a second intracellular pigment initiates light adaptation. To explain the observations in terms of the two-pigment hypothesis, one would have to postulate that the manipulations that lowered and then restored plasma-membrane rhodopsin concentration caused parallel concentration changes in the hypothesized intracellular pigment. This possibility has not been absolutely ruled out, but it is highly unlikely in view of the fact that during the pigment-shifting procedure an intracellular pigment would be subjected to a much smaller change in pH than the plasma-membrane pigment (Fig. 12).
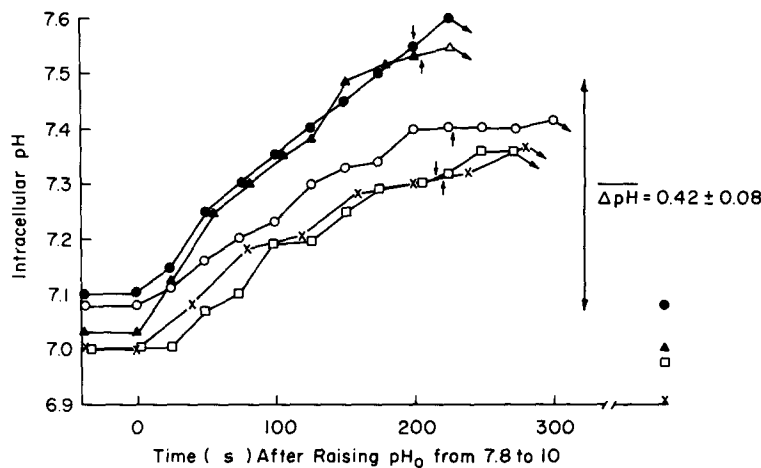

FIGURE 12.   Intracellular pH (pH$_i$) as a function of time after raising pH$_o$ from 7.8 to 10.0 in five cells. At t = 0, pH 10 superfusate arrives at the cell. At the arrows at t = 200 s, pH 7.8 superfusate arrives at the cell. The average rise in pH$_i$ caused by exposure to pH 10 superfusate was 0.42 ± 0.08 SD. The points at the far right are the steady-state pH to which pH$_i$ returns after the exposure to pH 10 superfusate.

The evidence against initiation of light adaptation by an intracellular pigment is based on experiments in the low and moderate intensity range (background lights not brighter than 3 × 10^{-5} W/cm^2) and therefore does not exclude a role for an intracellular pigment at high light intensities. This qualification is of particular importance in view of the possibility that bright lights induce vesiculation in some invertebrate photoreceptors (White and Lord, 1975). A second qualification relates to the possibility that a small component of light adaptation might be initiated by an intracellular pigment. The agreement between Δlog 1/S_D and Δlog I_2 (Table I) is not sufficiently good to rule out the possibility that an intracellular pigment is responsible for up to 5% of light adaptation (this calculation assumes that the postulated intracellular pigment has a concentration that remains constant when the rhodopsin concentration is changed). Finally, our results in no way rule out the possibility that separate populations of plasma-membrane rhodopsin initiate excitation and light adaptation.

Because the most obvious pigment-bearing membranes in invertebrate photoreceptors are the microvilli, which are infoldings of the plasma membrane, it has been commonly believed that visual pigment in the plasma membrane initiates excitation. Our results lend experimental support to this notion. In particular, we have shown that the absolute sensitivity of the cell rises and falls with the concentration of rhodopsin as assayed by the ERP. Because the ERP is generated by the plasma membrane (Smith and Brown, 1966), it seems likely that plasma-membrane rhodopsin initiates excitation. The functional relationship between rhodopsin concentration and absolute sensitivity appears to be linear (Table I), but our method of measuring rhodopsin concentration is not sufficiently accurate to rule out small nonlinearities. Data from other invertebrate photoreceptors also support a linear relationship between rhodopsin concentration and absolute sensitivity (Hamdorf et al., 1973; Rosner, 1975). In contrast, Minke et al. (1974) report that the sensitivity of partially dark-adapted barnacle photoreceptors is independent of rhodopsin concentration.

Our results do not support a physiological role for metarhodopsin in the intensity range we have examined. In both low and high rhodopsin states, a substantial fraction of visual pigment is in the metarhodopsin state (approximately 90% in the low rhodopsin state, and 50% in the high rhodopsin state). Thus much of the incident light is absorbed by metarhodopsin and these absorptions could, in principle, activate physiological processes. However, as both excitation and adaptation parameters change in proportion to the change in rhodopsin concentration (a factor between 4 and 5) and not in proportion to the change in metarhodopsin concentration (a factor slightly >0.5), it would appear that the absorption of light by metarhodopsin does not initiate excitation or light adaptation. This conclusion is consistent with results in barnacle photoreceptors where the action spectra of both light adaptation and excitation closely match the absorption spectrum of rhodopsin (λ_{max} 530 nm) and are clearly different from the absorption spectrum of metarhodopsin (λ_{max} 495 nm) (Strong and Lisman, 1978). At very high light intensities metarhodopsin terminates afterpotentials initiated by rhodopsin in barnacle (Hochstein et al., 1973), fly (Tsukahara et al., 1977), and possibly also in *Limulus* ventral photoreceptors (Lisman and Sheline, 1976). The relationship of this antagonistic process to light adaptation remains unclear.

In summary, at low and moderate light intensities, the only active pigment appears to be plasma-membrane rhodopsin. There is no evidence to suggest the involvement of a second intracellular pigment or of metarhodopsin, the photoproduct of plasma-membrane rhodopsin.

Implications for Mechanism of Ca^{++} Release

There is considerable experimental support for the hypothesis that light adaptation in *Limulus* is mediated by a light-induced rise in intracellular Ca^{++} and that much of this Ca^{++} is released from an intracellular compartment (see Introduction). The results presented here make it unlikely that calcium release is initiated by a specialized visual pigment in the membrane of the intracellular store. Rather, it appears that a message of some sort must be sent from the rhodopsin in the plasma membrane to the intracellular store. The already

available data place strong restrictions on the possible nature of this message. In the first place, the message cannot be electrical, because depolarization of the plasma membrane does not bring about significant light adaptation (Fein and DeVoe, 1973), and because measurement of light-induced intracellular Ca^{++} release shows that it occurs even when the cell is voltage-clamped (Brown and Blinks, 1974). Secondly, the influx of ions that occurs as a result of excitation cannot be the primary stimulus for intracellular Ca^{++} release. The entry of magnesium cannot be important because its removal from the extracellular space has almost no effect on the waveform of responses to adapting lights (Lisman, 1971). The entry of calcium cannot be an important stimulus for calcium release because the release occurs even when extracellular calcium is removed (Brown and Blinks, 1974; Lisman, 1976). The entry of sodium through the light-activated channels may be an important event in initiating a component of light adaptation (Lisman and Brown, 1972 b), but its role is probably limited to high light intensities. Injection of Na^+ ions leads to a desensitization (Lisman and Brown, 1972 a) and to a rise of Ca_i (Waloga et al., 1975). Na^+ injection produces no desensitization when extracellular calcium is removed (Lisman and Brown, 1972 a), and it therefore seems likely that sodium reduces sensitivity by affecting Ca^{++} transport across the plasma membrane. This mechanism cannot be the major mechanism of light-induced desensitization, because removal of external Ca^{++} has no effect on light-induced desensitization at low and moderate adapting intensities (Lisman, 1976). Furthermore, the recovery of sensitivity after sodium injection has a time-course quite different from the recovery following an adapting stimulus (Fein and Charlton, 1977 b).

The remaining possibility is that an intracellular messenger sent from the plasma membrane modulates Ca^{++} release from the intracellular compartment. There is currently no evidence for such a transmitter. However, the ability of a single rhodopsin molecule to cause a conductance change of 50 nmho, which is orders of magnitude larger than the conductances of known biological channels, has led to the postulation (Cone, 1973) of a chemical transmitter taking part in the excitation process. If this view is correct, it is plausible that a similar or even identical transmitter, released from the plasma membrane by rhodopsin, could trigger Ca release from the intracellular compartment and thereby cause light adaptation. This model is schematized in Fig. 13.

The intracellular compartment that releases Ca^{++} in the light may be the subrhabdomeric cisternae. These structures, which are seen in most invertebrate photoreceptors, are located in the light-transducing regions of the cell. Recent results in the honeybee demonstrate that the cisternae contain a high Ca^{++} concentration (Perrelet and Baede, 1978), but there are other possible Ca^{++} releasing compartments, including the plasma membrane itself. If light adaptation were mediated by Ca^{++} released from the plasma membrane, it seems likely that a message would be needed to couple the isomerization of a rhodopsin molecule to the release of Ca from a large area of the membrane. Light adaptation can occur after the absorption of 10–100 photons (Lisman, 1971). For such stimuli to change Ca_i by 10^{-8} M, each activated rhodopsin would have to release between 10^4 and 10^5 Ca^{++}. This is more than one would expect to be bound to a single protein molecule.

Implications for Visual Pigment Studies

Our ability to produce changes in the absolute sensitivity by manipulation of light and pH lends support to the conclusion that *Limulus* rhodopsin is very similar to squid rhodopsin, a conclusion derived previously from analysis of ERPs. Perhaps the most striking observation is that, under certain conditions, long wavelength irradiation leads to a rise in absolute sensitivity. Inasmuch as excitation is itself mediated by a long wavelength pigment (λ_{max} 525), this result would a priori be quite inexplicable, but follows in a straightforward fashion for a pigment with the properties of squid visual pigment.
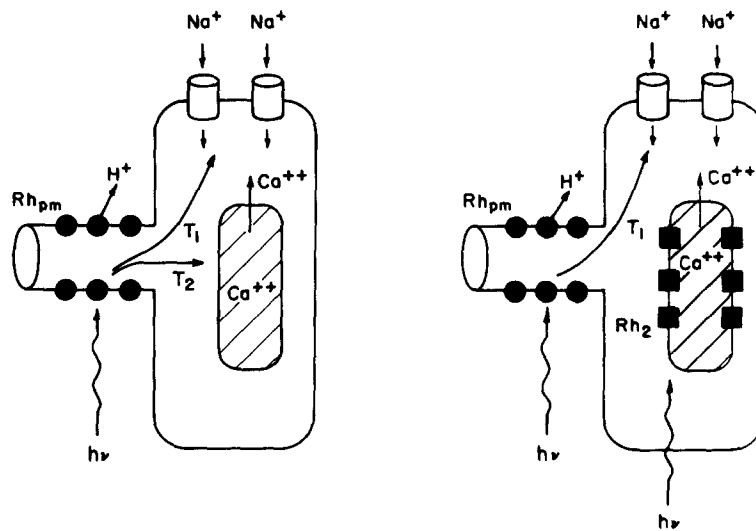

FIGURE 13. Schematic models for the (left) single- and (right) dual-pigment models. The hatched area represents an intracellular calcium-containing compartment, which, in the two-pigment model, contains a visual pigment (Rh_2). In both models the microvilli of the plasma membrane contain visual pigment (Rh_{pm}) which initiates excitation (the opening of sodium channels). The channels are coupled to Rh_{pm} by an intracellular transmitter (T_1). Intracellular free Ca somehow modulates this coupling, thereby producing light adaptation. The single-pigment hypothesis requires that Rh_{pm} send a transmitter, T_2, to the intracellular compartment to cause Ca^{++} release. In the dual-pigment model, Ca^{++} release is initiated by Rh_2.

The function of a possible second visual pigment in *Limulus* remains mysterious. The existence of a second visual pigment is suggested by spectrophotometric studies of the ventral eye. The bleaching characteristics and sensitivity to external pH of the pigment observed spectroscopically appear fundamentally different from the pigment observed using the ERP (see Lisman and Sheline, 1976). The ERP is a voltage measured across the plasma membrane and is therefore only due to pigment in the plasma membrane. The most likely resolution of the ERP and spectrophotometric results is that the ventral eye contains two pigments, one in the plasma membrane similar to squid rhodopsin, and one in the interior which does not contribute to the ERP, but which,

because of its high concentration, dominates the spectrophotometric observations. This pigment appears to have many characteristics in common with squid retinochrome, whose function is also mysterious.

Conclusions

Both excitation and light adaptation are initiated by rhodopsin in the plasma membrane. On the likely assumption that Ca^{++} release from an intracellular store is important in the physiology of light adaptation, it must be assumed that an intracellular message sent from the plasma membrane activates Ca^{++} release from the intracellular store. Biochemical approaches may therefore prove fruitful for further elucidation of the mechanism of light adaptation.

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