The Initial Response of *Limulus* Ventral Photoreceptors to Bright Flashes

Released Calcium as a Synergist to Excitation

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ABSTRACT   The leading edge of the response of *Limulus* ventral photoreceptors to brief flashes was investigated using a voltage clamp. The leading edge of responses increases linearly with flash intensity when dim flashes produce less than one photoisomerization per square micron of cell surface. Brighter flashes accelerate the initial portion of the response, resulting in a fourth-power relationship between the magnitude of the response at brief times after the flash and the flash intensity. The onset of this nonlinearity with increasing flash intensity is determined by the local density of photoisomerizations within the receptor. Responses to bright 10–15-μm-diam spots therefore rise faster than responses to diffuse flashes producing the same number of photoisomerizations within the receptor. Background illumination shortens the response latency and suppresses the initial nonlinearity. These phenomena can be explained by a model of transduction in which light activates two parallel cascades of reactions. Particles released by the first of these cascades open ionic channels, while the second produces an agent that accelerates the rate of production of particles by the first. Injection of the calcium buffer EGTA slows the initial portion of the response to bright flashes and suppresses its nonlinearity, which suggests that the accelerating agent released by the second cascade is calcium.

INTRODUCTION

After the absorption of a photon, the response of photoreceptors rises after a delay. This delay is thought to be caused by a cascade of chemical reactions that is initiated by the photoisomerization of a rhodopsin molecule, Rh* (Wald, 1965). These reactions are thought to lead to the production of "internal transmitter" molecules, which modulate the conductance of the photoreceptor's plasma membrane (see Cone, 1973). For *Limulus* ventral photoreceptors, illumination results in the flow of current into the cell because of an increased membrane conductance (Millecchia and Mauro, 1969b).

In the study of invertebrate photoreception, several models of phototransduction have assumed linear biochemical mechanisms for the steps leading to the activation of an inward current (Fuortes and Hodgkin, 1964; Borsellino et al., 1965; Levinson, 1966; French, 1980). These models described excitation as a single cascade of first-order reactions. No cooperative action by the products of the cascade, or by the products of parallel cascades initiated by a photoisomerization, was considered. The response of these models shortly after a flash of light therefore initially increased linearly with increasing flash intensity.

A choice between these linear and other, nonlinear models of excitation (Hamdorf and Kirschfeld, 1980; Payne and Howard, 1981) could be made if the initial linearity of the response of a voltage-clamped invertebrate photoreceptor were established over a wide range of flash intensities (see Baylor et al., 1974). Because the activating intermediates may only diffuse a few microns from the site of a photoisomerization (Lamb et al., 1981), it is necessary to demonstrate that the response is initially linear for flashes that produce several isomerizations per square micron of cell surface.

We demonstrate that when flashes isomerize more than one rhodopsin per square micron, the response of dark-adapted ventral photoreceptors is not linear with flash intensity, but it initially rises as the fourth power of the flash intensity. This nonlinearity of the initial response with flash intensity is suppressed by background illumination and by the intracellular pressure injection of a mixture of calcium and the calcium buffer EGTA. We propose that the nonlinear nature of the initial response of dark-adapted receptors to bright flashes is a result of the rise in the intracellular free calcium concentration, Ca_i, that follows a flash (Brown and Blinks, 1974). The initial kinetics of the response to bright flashes can be modeled on the assumption that a release of calcium by light initially accelerates the rates of the reactions that increase the membrane conductance. The initial response is then the cooperative outcome of two parallel chains of reactions, one chain leading to the release of calcium, the other leading directly to the increased membrane conductance. Before desensitizing the receptor (Brown and Lisman, 1975), released calcium may therefore act as a synergist to visual excitation.

METHODS

Electrical Recording

The methods used were very similar to those first described by Millecchia and Mauro (1969a, b). Briefly, ventral nerves of Limulus polyphemus were dissected and prepared as described by Fein and DeVoe (1973). Nerves were mounted on a transparent Sylgard base in a Plexiglas chamber and bathed in artificial seawater buffered to pH 7.0 with 10 mM HEPES. Photoreceptors that appeared to be isolated from their neighbors were impaled with two glass micropipettes of resistance 10–15 MΩ filled with 2.5 M KCl. A voltage-clamp circuit, described by Fein and Charlton (1977a), was used to record the photocurrent. Cells were voltage-clamped to their resting (dark) potential throughout the experiment. Before clamping the membrane potential, the light responses measured through the electrodes were compared to check for isopotential recordings. Photocurrents were digitized and averaged by a MACSYM 2 computer (Analog Devices, Norwood, MA).

Stimulation

Photoreceptors were stimulated using an optical bench with two light beams and a common light source, described by Fein and Charlton (1975b). Identical circular neutral density (ND) wedges and ND filters were used to attenuate the beams. An infrared blocking filter (BG-18, Schott Optical Glass, Inc., Duryea, PA) was placed between the preparation and the stimulating light beams. For experiments (Fig. 1) requiring diffuse monochromatic light, 10-nm-bandwidth interference filters, a heat filter (KG-3, Schott Optical Glass, Inc.), and an ultraviolet absorbing filter (2N460, Baird-Atomic, Bedford, MA) were placed in the path of the stimulating beam. Light from the two beams was combined and focused onto the preparation from below using a 10× microscope objective. Field stops were placed in the path of the two beams and focused onto the preparation so as to limit the area illuminated by one beam to a 600-μm-diam circle of diffuse light and by the other to a 5-μm-diam spot.

The preparation was viewed from above at a magnification of 320. Because the illumination was delivered from below, we could visually monitor the scatter and the focus of the light spot. For the experiments using the spot of light, we chose to record from photoreceptors on the edge or side of the ventral nerve, which could be illuminated without passing light through the nerve (Fein and Charlton, 1975b). Experiments were abandoned if the observed diameter of the spot of light was increased to >20 μm by scatter.

A single 45-W tungsten-halogen lamp (Sylvania, Danvers, MA) delivered white light of unattenuated intensity 6 mW/cm^2 in the plane of the preparation through the infrared filter. Illumination of the ventral photoreceptors by the diffuse source, attenuated by ~7.5 log units, elicited an average of one quantum bump per second. Illumination by the 5-μm-diam spot, attenuated ~5.5 log units, elicted one discrete wave per second.

For very bright flashes, small xenon flash lamps (Sunflash 133 Thyristor, Sunpack Corp., Woodside, NY) were used as alternative sources for each beam. For each cell and each beam, the intensity of the xenon flash was calibrated relative to that of the tungsten source by a physiological measurement. We first estimated the number of effective photons delivered by the tungsten source by determining the attenuation that elicited approximately one discrete wave per second during steady illumination of a photoreceptor. One quantum bump was assumed to indicate one effectively absorbed photon (Lillywhite, 1977). We then measured the attenuation of the xenon flash lamp that produced the same amplitude photocurrent as a 10-ms flash from a known attenuation of the tungsten source. We could then calculate the number of effective photons delivered by the flash lamp. Use of the xenon lamp is noted in the figure legends where applicable.

In some experiments, the impalement and illumination of the photoreceptor was visually monitored using an infrared-sensitive television camera (Dage MTI, Inc., Michigan City, IN) with an Ultricon tube (RCA, Lancaster, PA). Continuous infrared light was then focused onto the preparation through a third light beam with an independent source.

Pressure Injection of Substances into Cells

Instead of the KCl-filled micropipette usually used to monitor the membrane potential, a blunter micropipette containing the substance to be injected was used to impale the cell, both to monitor membrane potential and to pressure-inject. This method of injection is identical to that of Corson and Fein (1983).

Chemicals

HEPES and EGTA (Sigma Chemical Co., St. Louis, MO) were neutralized to pH 7.0 with KOH. To make up the CaEGTA solutions that were injected into cells, Ca(OH)$_2$ was

dissolved in K_2EGTA and the pH was subsequently adjusted to 7.0. The free calcium concentration of these solutions was measured with a calcium-sensitive macroelectrode (Lanter et al., 1982).

THEORY

The dimensions of constants and variables used in this paper are listed in Table I and after each introduction in the text.

Linear Visual Cascades

Several authors have modeled phototransduction with a cascade of first-order chemical reactions (e.g., Borsellino et al., 1965; Baylor et al., 1974). Light initiates the cascade by the production of particles y_1 (mol) that, in turn, initiate the production of particles y_2, etc., via first-order reactions. The final particle in the cascade, y_n, modulates the membrane conductance. All particles decay via first-order reactions.

Such a scheme is realized by the equations:

$$\frac{dy_1}{dt} = caI(t) - \alpha_1 y_1;$$

$$\frac{dy_2}{dt} = \gamma_1 y_1 - \alpha_2 y_2;$$

$$\frac{dy_3}{dt} = \ldots\ldots\ldots;$$

$$\frac{dy_n}{dt} = \gamma_{n-1} y_{n-1} - \alpha_n y_n,$$

(1)

where $\gamma_1 \ldots \gamma_{n-1}$ are first-order forward rate constants (s^{-1}) producing particles and $\alpha_1 \ldots \alpha_n$ are first-order rate constants (s^{-1}) of decay of particles. a is the effective area of the cell illuminated (μm^2) and c is the efficiency of the first reaction $(mol \cdot photon^{-1})$. $I(t)$ $(photons \cdot \mu m^{-2} \cdot s^{-1})$ describes the light flux.

At small values of the time after a brief flash, when the rates of decay of particles at all stages are much less than their rates of production, Eq. 1 predicts an initial response given by

$$y_n = CNt^{n-1}/(n - 1)!$$

(2)

C $[mol \cdot photon^{-1} \cdot s^{-(n-1)}]$ is a constant proportional to the product of the rate constants, γ, for the production of particles in the cascade.

$$C = c \prod_{r=1}^{n-1} \gamma_r.$$

N (photons) is the effective quantum content of the flash of intensity, I, and duration Δt.

$$N = aI\Delta t.$$

Eq. 2 results in a response that initially rises as t^{n-1} at a rate that is independent of the decay rate constants and is in linear proportion to the flash intensity.

At later times in the response to a flash, the decay of particles in the cascade becomes significant and determines the amplitude, time to peak, and duration of the response. An example is a reaction scheme similar to that investigated by Baylor et al. (1974), in which $n - 1$ of the particles decay with the rate α and one particle decays with a slower rate, $\alpha\beta$ (β, dimensionless).

$$\gamma_1 = \gamma_2 = \ldots = \gamma_{n-1} = \gamma;$$

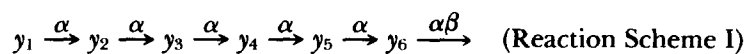$$\alpha_1 = \alpha_2 = \ldots = \alpha_{n-1} = \alpha;$$

$$\alpha_n = \alpha\beta.$$

TABLE I
Constants Used in Models

Constant	Description	Dimension
(1) Linear models		
c	Quantum efficiency of production of transmitter	$mol \cdot photon^{-1}$
a	Area effectively illuminated	μm^2
α, γ	First-order rate constants	s^{-1}
C	Product of rate constants, γ_n, and quantum efficiency, c	$mol \cdot photon^{-1} \cdot s^{-(n-1)}$
β	Ratio of rate constants	—
(2) Additional constants used in the nonlinear model		
K	Second-order rate constant	$mol^{-1} \cdot s^{-1}$
C_x	Product of rate constants and quantum efficiency for production of x	$mol \cdot photon^{-1} \cdot s^{-4}$
A^3	Magnitude of linear component of initial response	$mol \cdot photon^{-1} \cdot s^{-5}$
B^3	Magnitude of nonlinear component of initial response	$mol \cdot photon^{-4} \cdot s^{-17}$
k	Fraction of area, a, for effective summation of accelerating agent	—
i_{sat}	Saturating photocurrent	nA
σ	Binding constant of transmitter to channels	mol
s	Sensitivity of photocurrent to transmitter, y (equal to i_{sat}/σ)	$nA \cdot mol^{-1}$

The output of such a cascade to a brief flash of light is given by:

$$y_n = CN(\alpha - \alpha\beta)^{-(n-1)} \left[e^{-\alpha\beta t} - e^{-\alpha t} \sum_{r=0}^{n-2} \frac{(\alpha - \alpha\beta)^r t^r}{r!} \right]. \tag{3}$$

Baylor et al. (1974) investigated a specific case of this cascade, in which $\gamma = \alpha$ and $n = 6$, in order to describe the response of cone photoreceptors. The cascade was realized by the reaction scheme

$$y_1 \xrightarrow{\alpha} y_2 \xrightarrow{\alpha} y_3 \xrightarrow{\alpha} y_4 \xrightarrow{\alpha} y_5 \xrightarrow{\alpha} y_6 \xrightarrow{\alpha\beta} \quad \text{(Reaction Scheme I)}$$

Nonlinear Visual Cascades

The earliest studies of the kinetics of phototransduction (Fuortes and Hodgkin, 1964; Borsellino et al., 1965) recognized that linear reaction schemes do not describe the response to bright flashes or the effects of background illumination. To explain the more rapid falling phase of responses to bright flashes and desensitization of the response to flashes superimposed upon backgrounds, it was suggested that light initiates a delayed increase in the rate constants of decay of the particles in the cascades described by Eq. 1 (Borsellino et al., 1965; Baylor et al., 1974). This proposal results in a delayed reduction in the amplitude of the reaction scheme's response, but it leaves the initial response of the cascade unaffected by bright flashes and backgrounds because Eq. 2 is not dependent on the rates of decay of the particles. Eq. 2 should therefore model the initial response to a flash under all illumination conditions, as has been demonstrated in vertebrate cones and rods (Baylor et al., 1974, 1979).

The results of the present paper show that Eq. 2 is not an adequate description of the initial response of ventral photoreceptors. Bright flashes and backgrounds increase the initial response far more than that expected from linear summation, thus shortening the apparent latency of the response (Millecchia and Mauro, 1969a). We now investigate the theoretical consequences of the proposal that this new nonlinear behavior of the initial response is caused by the delayed release by light of an agent x (mol), which increases the forward rate constants, γ, of the transduction cascade.
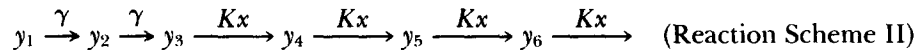
We shall consider a visual cascade with six stages, y_1-y_6. Two of the forward rate constants are unaffected by the agent x, and the remaining three are proportional to the effective quantity of x in the locality of the photoisomerization.

$$\gamma_1 = \gamma_2 = \gamma; \tag{4}$$

$$\gamma_3 = \gamma_4 = \gamma_5 = Kx(I, t), \tag{5}$$

where K is a second-order rate constant $(\text{mol}^{-1} \cdot \text{s}^{-1})$.

A possible reaction scheme, for example, is

$$y_1 \xrightarrow{\gamma} y_2 \xrightarrow{\gamma} y_3 \xrightarrow{Kx} y_4 \xrightarrow{Kx} y_5 \xrightarrow{Kx} y_6 \xrightarrow{Kx} \qquad \text{(Reaction Scheme II)}$$

To model the behavior of such a scheme following a brief flash, we must first describe the initial time course of the delayed release of x by light. We assume that x is released by a linear cascade of reactions, described by Eq. 1, that is independent of the cascade of reactions that produce y_6. If there are, for example, five cascaded elements producing x, then the total quantity of x initially released by a flash will be given by Eq. 2.

$$x_{\text{total}} = C_x N t^4 / 4!$$

Because the model is nonlinear, we must make assumptions about the spatial localization of x. We assume that the total quantity of x released by a given photoisomerization is effective upon the reactions that are initiated by that

photoisomerization. In addition, a fraction k of the total quantity of x released by other photoisomerizations in the flash is also effective at a given photoisomerization. The quantity of x, x_{flash}, that is initially effective after a flash at a given photoisomerization is therefore

$$x_{flash} = (1 + kN)C_x t^4/4! \qquad (k \ll 1),\tag{6}$$

where $(1 + kN)$ has the dimension photons and k (dimensionless) can be regarded as the fraction of the total area illuminated, a, over which effective summation of x occurs. To Eq. 6 we also add a quantity, x_0, which is the background quantity of x. The quantity of x initially effective on the reactions producing y_6 is therefore

$$x(I, t) = x_{flash} + x_0,$$

so that

$$\gamma_3 = \gamma_4 = \gamma_5 = K(x_{flash} + x_0).\tag{7}$$

We can now integrate the response of Reaction Scheme II at early times after a flash. We substitute Eq. 6 into Eq. 7 and then substitute Eq. 7 into Eq. 1. If we ignore the decay of particles at early times, integration of Eq. 1 predicts an initial response that is polynomial in x_0 and x_{flash}.

$$y_6 = cN\gamma^2 K^3 t^5 \left[\frac{x_0^3}{5!} + \frac{x_0^2 x_{flash}}{126} + \frac{x_0 x_{flash}^2}{364} + \frac{x_{flash}^3}{2,856} \right],\tag{8}$$

which is approximately equal to

$$y_6 = cN\gamma^2 K^3 t^5 \left[\frac{x_0}{(5!)^{1/3}} + \frac{x_{flash}}{(2,856)^{1/3}} \right]^3.$$

We can collect constants and substitute for x_{flash} to produce the final equation that describes the initial response.

$$y_6 = Nt^5 [A + B(1 + kN)t^4]^3,\tag{9}$$

where

$$A = Kx_0 (c\gamma^2/5!)^{1/3}$$

and

$$B = \frac{KC_x}{4!} (c\gamma^2/2,856)^{1/3}.$$

The constants A and B determine the respective magnitudes of the linear and nonlinear components of the initial response. The linear component is similar to Eq. 2.

$$y_6 = NA^3 t^5.\tag{10}$$

The nonlinear term dominates at high light intensities, when the initial response will approach the following form:

$$y_6 = N^4 k^3 B^3 t^{17}.\tag{11}$$

(The dimensions of A^3 and B^3 are mol·photon^{-1}·s^{-5} and mol·photon^{-4}·s^{-17}, respectively.)

For a given light intensity, the form of the initial response depends on the ratio of B to A. The constants A and B are determined by the relative magnitudes of x_0 and x_{flash}.

$$\frac{x_{flash}}{x_0} = \frac{B(1 + kN)t^4}{A} \cdot \left(\frac{2,856}{5!}\right)^{1/3}. \tag{12}$$

If the initial release of x, x_{flash}, dominates over the background quantity, x_0, then $B(1 + kN)t^4$ dominates in Eq. 9, resulting in an initial response that rises as the 17th power of the time after the flash. On the other hand, elevation of the background quantity of x to an extent where A dominates in Eq. 9 results in an earlier phase to the response that rises as the fifth power of the time after the flash and in linear proportion to the flash intensity.

Equations similar to Eqs. 8 and 9 can be derived for a variety of reaction schemes involving different numbers of parallel chains and particles. The choice of Reaction Scheme II to describe the initial response of ventral photoreceptors is dictated by the following considerations from the Results (see below). (a) Under conditions for which the response to bright flashes rises linearly with flash intensity (when light-adapted or after EGTA injection), the response rises as the fifth or sixth power of the time after the flash. The linear pathway of transduction producing particles y requires six to seven cascaded stages (Eq. 2) to reproduce this behavior. (b) In the dark-adapted state, when the response to bright flashes rises nonlinearly with flash intensity, the initial response at fixed times after the flash rises as the fourth power of flash intensity. If the accelerating agent, x, is produced in proportion to the flash intensity, then it must act at three sites in the cascade producing y_6 to reproduce this fourth-power relationship. (c) These nonlinear initial responses rise as the 17th power of the time after the flash. x must rise as the fourth power of time at each of its sites of action to reproduce this behavior. Therefore, x behaves as if it were produced by a parallel cascade having five cascaded stages.

RESULTS

Response of a Dark-adapted Photoreceptor to a Diffuse Flash of Light

Fig. 1 superimposes the response of a dark-adapted ventral photoreceptor to a single flash of dim, diffuse, monochromatic light, delivering an average of 10 effective photons, on the response to a bright single flash delivering 1,000 effective photons. The bright flash was delivered to the dark-adapted cell after the dim flash. In Fig. 1, the amplitude of the response to the brighter flash is reduced by a factor of 100; hence the dual labeling of the vertical axis. If the response of the photoreceptor were linearly proportional to the light intensity, the responses to the two flashes would superimpose at all times. This was not the case. The response to the brighter flash rose much faster than expected from linearity and it decayed faster. The faster decay of the response may be due to an acceleration by intense flashes of the rates of decay of intermediates in the

visual cascade (Fuortes and Hodgkin, 1964). In the present paper, we shall not consider these nonlinearities in the later part of the response. The experiments described in this paper are designed to quantify the nonlinearity with light intensity of the leading edge of the response, which we attribute to acceleration of the rates of production of the intermediates in the visual cascade (see Theory).

Initial Response of a Dark-adapted Photoreceptor to a Spot of Light

In this section, we describe the response of the voltage-clamped ventral photoreceptor to a flash of light delivered as a 5-μm-diam spot centered on the most sensitive region of the photoreceptor. The use of a spot of light allowed us to deliver a high density of effective photons, while minimizing the total photon flux. This reduced the peak photocurrent for intense flashes, thus improving the accuracy of the voltage clamp. The cells were found to dark-adapt faster after an intense flash delivered as a spot, rather than as diffuse light, which reduced the time taken for the experiment.
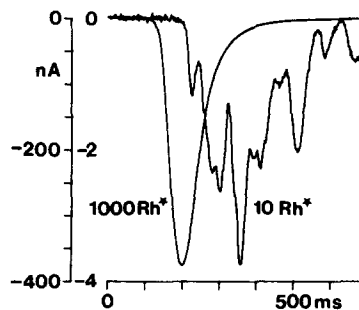

FIGURE 1.   Responses of a dark-adapted photoreceptor to diffuse flashes of 10 ms duration, wavelength 600 nm, delivering 10 and 1,000 effective photons. The response to 1,000 effective photons has been reduced in scale by a factor of 100.

Photoreceptors were dark-adapted for 45 min after impalement and dark-adapted between flashes. Dark adaptation between flashes was assessed from the amplitude, time scale, and latency of the response to a dim test flash. The experiment was terminated if the latency or amplitude drifted appreciably. Deterioration of the preparation was usually marked by an increase in latency and a reduction in the amplitude of the responses to dim flashes.

Responses to intensities containing fewer than 1,000 effective photons were averaged so as to maintain the same total quantal capture. 100 responses to 10 effective photons, 10 of the responses to 100 effective photons, etc., were averaged.

Fig. 2, A and B, illustrates average photocurrents generated by brief flashes of increasing intensity. For C and D, each response was divided by the corresponding flash intensity, and the result is plotted on an expanded time scale, so as to emphasize the leading edge of the responses. The responses in Fig. 2A to flashes delivering fewer than 100 effective photons coincide after scaling to produce Fig. 2C, which demonstrates the initial linearity of transduction for these inten-

sities (Lisman and Brown, 1975a). However, the traces of Fig. 2D, which are derived from the responses to the more intense flashes of Fig. 2B, do not coincide. As the flash intensity increases, the responses rise from the baseline at earlier times. Bright flashes therefore accelerate the initial response. The initial response to a 5-μm-diam spot of light exceeds the linear estimate when there are more than 100 effective photons in the stimulating beam. Since the 5-μm-diam
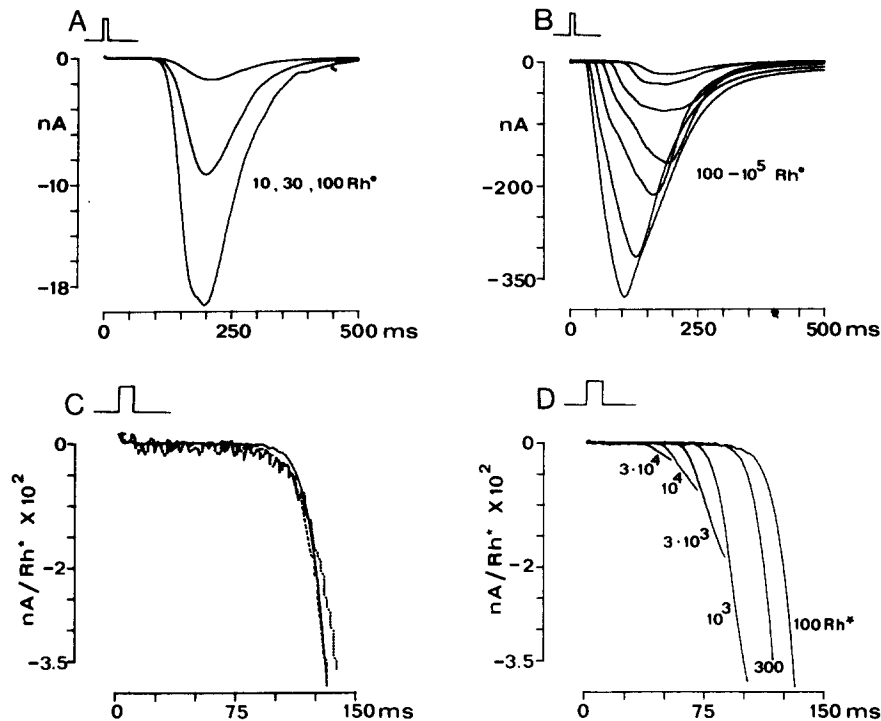

FIGURE 2.   (A and B) Average responses of a dark-adapted photoreceptor to flashes from a 5-μm-diam spot delivering (A) 10, 30, and 100, and (B) 100, 300, 1,000, 3,000, 10^4, 3×10^4, and 10^5 effective photons. Responses to fewer than 1,000 effective photons are averages of several presentations (see text). Flashes delivering 10^3 effective photons or fewer were of 10 ms duration, from a tungsten source. Those delivering $>10^3$ effective photons were from a xenon strobe (see Methods for details). (C and D) The initial portions of the responses of A and B, respectively, are divided by the number of effective photons delivered by the flash and plotted on an expanded scale. In C, the response to 10 Rh* is the dotted line, the response to 30 Rh* is the dashed line, and the response to 100 Rh* is the solid line.

spot is scattered to a 10–15-μm-diam spot by the nerve and photoreceptor, the delivery of 100 effective photons by the spot corresponds to a density of about one effective photon per square micron of the photoreceptor's surface.

Comparison of the Response to a Diffuse Light with That to a Spot

We wished to establish that the acceleration of the initial response to bright flashes is caused by an increased density of effective photons, rather than an

increased total number within the photoreceptor. Therefore, we compared the response to a spot of light with that to diffuse light delivering the same number of effective photons. The light-sensitive region (the R-lobe) of a large *Limulus* photoreceptor has an approximate diameter of 50 μm (Stern et al., 1982; Calman and Chamberlain, 1982) and so presents a cross-sectional area of ~2,000 μm^2 to diffuse illumination. A 5-μm-diam spot scattered to 10–15 μm diam, such as the one illuminating the photoreceptor of Fig. 3*A*, therefore covers between 1/10th
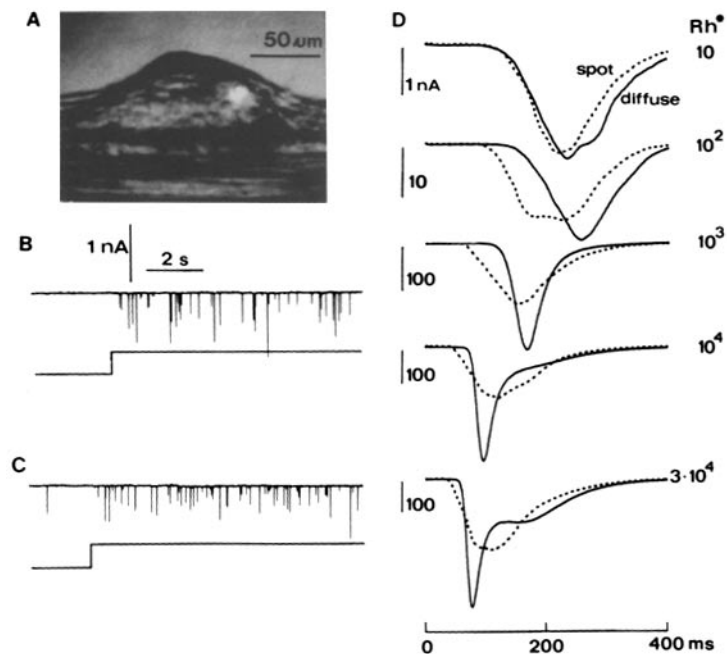

FIGURE 3. (*A*) Video recording of a ventral photoreceptor with a 5-μm-diam light spot centered on its most sensitive region. (*B*) Quantum bumps elicited by a step of diffuse light, intensity −7.6 log units. (*C*) Quantum bumps elicited by a step of light from the spot, intensity −5 log units. (*D*) Responses to flashes of increasing intensity delivered by diffuse light (solid line) or a spot (dotted line). The number of effective photons delivered by either light source is given on the right. 10-ms flashes from a tungsten source were used to deliver ≤10^3 effective photons. A xenon lamp (see Methods) delivered the brighter flashes. The responses were elicited in the order 10, 10^3, 100, 10^4, and 3 × 10^4 effective photons. For each intensity, the flash to the spot was delivered before that to the diffuse source.

and 1/30th of the R-lobe. If the R-lobe has uniform sensitivity, the spot will therefore deliver effective photons at a density 10–30 times higher than a diffuse light delivering the same total number of effective photons.

Photoreceptors were dark-adapted and illuminated by dim continuous illumination from either a 5-μm-diam spot or from a 600-μm-diam diffuse source (see Methods). The two beams were attenuated so as to elicit equal numbers of discrete waves (Fig. 3, *B* and *C*) when the receptors were continuously illumi-

nated. Once this balance was achieved, equal ND filters were removed from each beam and the average response to flashes of light of increasing intensity was obtained (Fig. 3D). The photoreceptors were dark-adapted between each flash.

Fig. 3D demonstrates that the leading edge of the average responses of the photoreceptor to either diffuse or spot illumination were very similar when 10 effective photons were delivered. This observation confirms the study of Spiegler and Yeandle (1974), in which no regional differences in transduction were discerned for dim illumination. However, when 100 or more effective photons were delivered, the response to the spot rose more rapidly and reached a smaller peak amplitude. Similar observations were made on four other cells. In the case of the experiment illustrated in Fig. 3, the single responses to flashes delivering 1,000 effective photons were recorded immediately after the averages of those to 10 effective photons. The response to the spot was recorded before that to the diffuse light. Neither the reduced latency of the response to the spot at the higher intensity nor its reduced amplitude could therefore be ascribed to light adaptation.

Fig. 3D demonstrates that, for flashes delivering more than 100 effective photons, the leading edge of the response is a function of the density of effective photons, as well as the total number delivered. The latency of the response to a spot delivering 1,000 effective photons lies between those of the responses to diffuse flashes delivering 10^4 and 3×10^4 effective photons. If the acceleration of the initial response to intense flashes is caused by an agent that diffuses from the site of one isomerization to a nearby one, then the results of this section tell us that this agent cannot immediately spread throughout the cell, but that it must be localized to an area illuminated by the spot. Immediate spread would sum the effects of even distant photons, so that the latency of the response to intense flashes would be independent of the local density of effective photons and dependent only on the total number falling on the whole cell. On the other hand, confinement of the agent to the area of the spot means that diffuse flashes must deliver a greater total number of effective photons to match the density of isomerizations in the spot and hence match the latency of the response to the spot.

Background Illumination Imposes Linearity on the Initial Response

Background illumination reduces the sensitivity and the latency of the average response of ventral photoreceptors to superimposed flashes of light (Millecchia and Mauro, 1969a). Wong et al. (1982) have demonstrated that the latency of quantal events is reduced by background illumination. This section investigates the effects of background illumination on the nonlinear kinetics of the initial response to superimposed bright flashes. Fig. 4, A and B, illustrates responses to the same flashes of increasing intensity, delivered as 5-μm-diam spots, when the cell was dark-adapted (Fig. 4A) and when the flashes from the spot of light were superimposed upon a diffuse background that delivered 1,000 effective photons per second (Fig. 4B). The background produced a steady inward current of 2 nA. Fig. 4, C and D, shows log-log plots of the response per effective photon vs. time during the response, calculated from Fig. 4, A and B, respectively. These
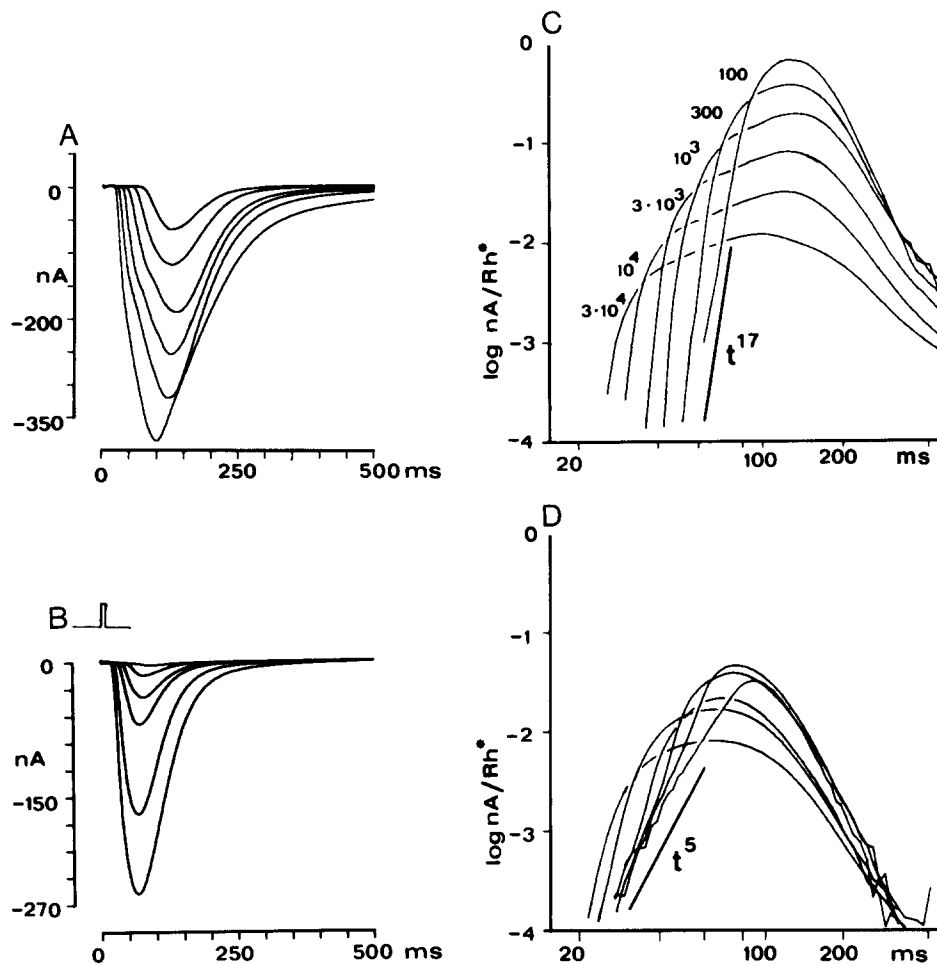
FIGURE 4. (*A*) Responses of a dark-adapted photoreceptor to 100, 300, 1,000, 3,000, 10^4, and 3×10^4 effective photons, delivered as flashes from a 5-μm spot of light centered on the cell's most sensitive region. Responses to 100 effective photons are the average of 10 presentations; those to 300 Rh* are the average of 3. Flashes delivering >1,000 effective photons were produced by a xenon strobe; lesser intensities were produced by a 10-ms flash from a tungsten source (see Methods). (*B*) Responses to the same flash intensity series as in *A*, when the receptor was light-adapted by a diffuse light source delivering ~1,000 effective photons per receptor per second. (*C*) The dark-adapted responses in *A* are divided by the number of effective photons in each flash and plotted on log-log axes. The curves are truncated at the level of the baseline noise for clarity. (*D*) The light-adapted responses to the flashes in *B* are divided by the number of effective photons and plotted on log-log coordinates. As the flash intensity increases, the rising edges of the log-log plots deviate progressively to the left.

plots illustrate the entire responses while emphasizing the initial portions. They also enable a direct comparison to be made with log-log plots of the responses of vertebrate rods and cones described by Baylor et al. (1974, 1979).

Two nonlinearities modify the rising edges of the dark-adapted responses, over the range of intensities delivered (100–3 × 10^4 effective photons). The initial response of the dark-adapted photoreceptor accelerates and begins to saturate. The onset of saturation is demonstrated in Fig. 4C by the reduction in the peak log response per effective photon with increasing flash intensity. Peak responses to flashes generating >70 nA increase by much less than the linear estimate. For diffuse illumination, Brown and Coles (1979) demonstrated that saturation does not begin to limit the peak photocurrent until >400 nA is generated, as is also seen in our Fig. 1. We attribute the much smaller value of 70 nA in this experiment to the use of a spot and note that this difference implies that the spread of activation is limited within the cell, as has been directly demonstrated by Fein and Charlton (1975a). However, even at the highest flash intensity, the response never fully saturates. Rather, the responses to the brightest flashes continue to increase slowly with flash intensity. This slow increase was also noted by Brown and Coles (1979).

The second nonlinearity shifts the steep rising edges of the log-log plots of Fig. 4C to earlier times, demonstrating acceleration of the initial response to these intense flashes, as in Fig. 2D. The initial log-log responses rise as an approximately straight line with a slope of ~17. Thus, the dark-adapted response to bright flashes is initially approximately proportional to the 17th power of the time after the flash.

Background illumination reduced the sensitivity of the receptor. The amplitude of the response to 100 effective photons was reduced from 70 to 3 nA. Several modifications in the time course of the response were produced by background illumination. First, the latency of the response was reduced. The time taken for the response to 100 effective photons to reach 10^{-3} nA per effective photon was reduced from 65 (dark-adapted) to 40 ms when light-adapted. Thus, although light adaptation reduced the peak response amplitude, it greatly increased the initial response at a fixed time after the flash. Second, the light-adapted responses to flashes delivering between 100 and 3,000 effective photons initially increased in linear proportion to the light intensity. This can be deduced from the initial superposition of the traces on the log-log plot of Fig. 4D. The responses to these flash intensities only began to increase more than linearly when later times in the response were considered. Responses to >3,000 effective photons, however, increased more than linearly at the earliest times for which current flow can be reliably detected. In the next section, we show that at fixed times shortly after the flash, the dark-adapted responses approached a fourth-power relationship to the flash intensity.

A third modification to the leading edge of the responses in the presence of the background is the reduction in the initial slope of the log-log plots of Fig. 4D when compared with those of Fig. 4C. Instead of rising as approximately t^{17}, the light-adapted response to 100 effective photons initially rose as t^5. This reduction reflects the less abrupt rising edge of the response when it is light-adapted.

Background illumination therefore reduced the latency of the responses to the dimmer flashes used in the experiment of Fig. 4. It also extended the range of intensities for which the initial response behaved linearly. The results of Fig. 4C were confirmed by plotting an intensity-response series from a total of eight dark-adapted cells on log-log coordinates. As in Fig. 4C, (a) dark-adapted responses to 5-μm-diam light spots delivering >100 effective photons rose approximately as the 17th power of the time after the flash (mean exponent; mean ± SD 15.8 ± 3.1), and (b) at fixed times shortly after the flash, the photocurrent approached a fourth-power relationship to the flash intensity (mean maximum exponent for each cell, 4.5 ± 1.1).

Six of the eight cells were light-adapted by diffuse backgrounds delivering 1,000 effective photons per second. Log-log plots of the intensity-response series revealed that, as in Fig. 4D, (a) a reduction in the response latency resulted in a large increase in the photocurrent flowing at early times when compared with dark-adapted responses; (b) this initial phase of the light-adapted response rose approximately as the fifth power of the time after the flash (mean exponent, 4.6 ± 0.7); (c) for the range of flash intensities over which the dark-adapted response rose as the fourth power of flash intensity, this initial phase of the light-adapted response rose more nearly linearly (mean exponent, 1.5 ± 0.31).

In the following section, we quantitatively examine the changes in the initial response that are due to light adaptation. The reduction in peak response amplitude, the reduced time to peak, and the faster decay of the light-adapted response are not considered. Fuortes and Hodgkin (1964) suggest that these are due to a faster rate of decay of intermediates in the visual cascade. Our analysis (see Theory) concentrates on the kinetics of production of intermediates in the cascade.

Intensity-Response Relationships at Fixed, Early Times After a Flash

Intensity-response relationships were plotted from the data of Fig. 4. They show the relationship between photocurrent and effective flash energy at several different fixed, early times in the dark-adapted state (Fig. 5A) and in the presence of diffuse background illumination that delivered 1,000 effective photons per second (Fig. 5B). Fig. 5C compares relationships between effective flash energy and response recorded 40 ms after the flash in the light- and dark-adapted states.

The continuous curves in Fig. 5 are solutions to the following equations, which relate the effective photons delivered by the flash, N, to the photocurrent, i_L:

$$y = Nt^5[A + B(1 + kN)t^4]^3; \tag{9}$$

$$i_L = yi_{sat}/(\sigma + y), \tag{13}$$

where i_{sat} is the photocurrent at saturation and σ is the quantity of y that produces 50% saturation. (The dimensions of i_{sat} and σ are amperes and moles, respectively.)

The constant A determines the magnitude of a response component that is linearly related to flash intensity, while B determines the magnitude of a nonlinear component, rising as the fourth power of the flash intensity.

A and B were chosen so as to best fit the data available. In the dark-adapted
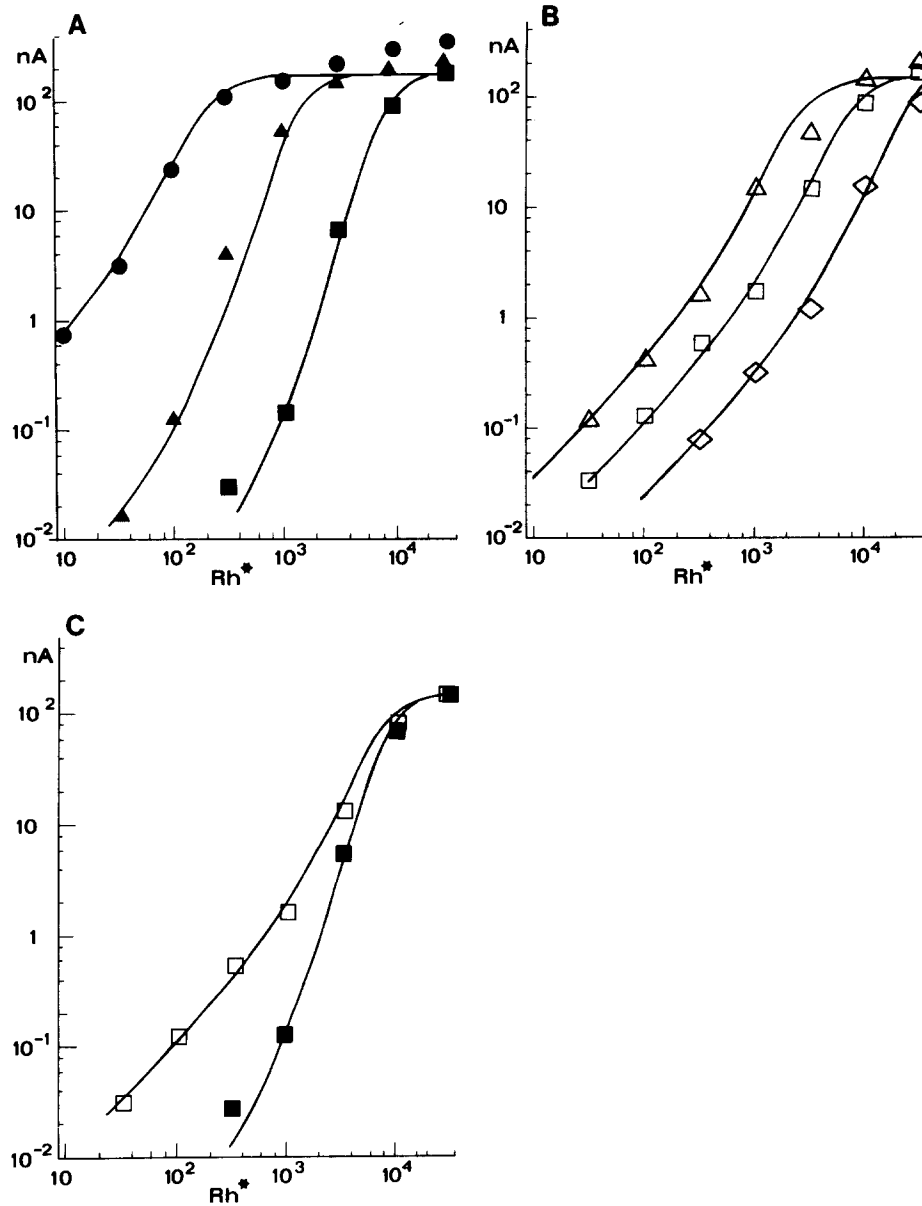
FIGURE 5.   Intensity-response relationships to light flashes plotted at fixed times after the flash (same cell as Fig. 4). (A) Dark-adapted relationships recorded 40 (squares), 60 (triangles), and 90 (circles) ms after the flash. The solid lines are solutions to Eqs. 9 and 13 with parameters: $A^3/\sigma = 0.63$ photon^{-1}·s^{-5}, $B^3/\sigma = 1.134 \times 10^{14}$ photon^{-4}·s^{-17}, $k = 9.1 \times 10^{-3}$, and $i_{sat} = 158$ nA. (B) Light-adapted relationships recorded 30 (diamonds), 40 (squares), and 50 (triangles) ms after the flash. The solid lines are solutions to Eqs. 9 and 13 with $A^3/\sigma = 61.6$ photons^{-1}·s^{-5} and the other constants as in A. (C) Light-adapted (open symbols) and dark-adapted relationships at 40 ms after the flash. Solid lines are solutions to Eqs. 9 and 13, as in A and B.

state, the term $B(1 + kN)t^4$ dominates in Eq. 9, resulting in responses that rise as the 17th power of the time after the flash and as the fourth power of the flash energy for the brightest flashes (Fig. 5, *A* and *C*). The effect of light adaptation is modeled by keeping *B* constant while increasing *A* by 4.6-fold, so as to best fit the data. At early times, *A* dominates in Eq. 9, resulting in an early phase to the response that rises as the fifth power of the time after the flash and is more nearly linear with flash intensity (Fig. 5, *B* and *C*).

Data from two other cells were well fitted with Eqs. 9 and 13. In both cases, the effect of a background illumination delivering 1,000 effective photons per second could be modeled by increasing *A* by 6.52-fold and 5.2-fold, respectively, while keeping *B* constant.

Physically, Eq. 9 could be regarded as describing the release, by a flash, of particles, *y*, that open ionic channels in the saturable manner described in Eq. 13. Eqs. 9 and 13 summarize two important features of the initial response. First, for photocurrents below saturation, the nonlinearity of the rising edge of the response does not depend on the photocurrent flowing, but on the intensity of the flash and the time after the flash. For example, Fig. 5*A* (circles) shows that, 90 ms after the flash, photocurrents between 0.8 and 3 nA rose almost linearly as the flash energy was raised from 10 to 30 effective photons. However, 40 ms after the flash (squares), the photocurrents rose through the same range of current much more than linearly as the flash intensity was raised from 1,000 to 3,000 effective photons. Second, for early times, saturation through Eq. 13 begins to limit the photocurrent at approximately the same photocurrent, irrespective of the time after the flash, the flash intensity, or the presence of an adapting background. However, although Eq. 13 adequately describes the onset of saturation, it is clearly not able to reproduce the continued slow rise of the current with intensity for very bright flashes (Brown and Coles, 1979).

In the theoretical section of this paper, we discuss the difference between Eq. 9 and the linear prediction of cascaded first-order reaction schemes (Borsellino et al., 1965; Baylor et al., 1974). We show that an equation such as Eq. 9 can be obtained if we assume that, in addition to initiating a visual cascade having six intermediates, light also initiates the delayed local release of an agent that accelerates three of those reactions in proportion to its concentration in the neighborhood of a photoisomerization. The term $B(1 + kN)t^4$ is proportional to the rise in the concentration of the accelerating agent after a flash. *A* is proportional to the background concentration of the accelerating agent (see Eq. 12). In our model, an approximately fivefold increase in the concentration of the accelerating agent will therefore mimic the effects of background on the initial response. The constant *k* in the model gives the effective area over which summation of the accelerating agent occurs, expressed as a fraction of the total area. The value of *k* for the cell of Fig. 5 is 0.0091, which is equivalent to ~1 μm^2, given a nominal spot diameter of 10 μm.

Eq. 12 gives the ratio of the concentration of the accelerating agent released by light in our model to the resting concentration of that agent. The substitution of the parameters used to model the data of Fig. 5 results in a ratio that exceeds unity 30 ms after a flash delivering 1,000 effective photons to a spot centered on the most sensitive region of the dark-adapted receptor. Significant photocur-

rent (>0.1 nA) is not generated until 40 ms have elapsed after the flash. In the light-adapted state, however, because of the higher resting concentration of the agent, the ratio exceeds unity 40 ms after the flash. A significant photocurrent is already generated 20 ms after the same flash. Thus, if a ratio of unity is arbitrarily taken as a threshold for the detection of the accelerating agent, then detection slightly precedes the generation of significant photocurrent in the dark-adapted state and lags in the light-adapted state.

Injection of CaEGTA Imposes Linearity on the Response to Bright Flashes

Bright flashes are known to cause a rapid increase in intracellular calcium (Brown and Blinks, 1974). Intracellular injection of calcium is also known to accelerate the initial response to flashes of light (Brown and Lisman, 1975). We therefore thought it possible that the acceleration produced by bright flashes was due to an early release of intracellular calcium. Injection of the calcium buffer EGTA suppresses the transient increase of intracellular free calcium that follows a flash (Brown and Blinks, 1974). We therefore decided to see whether intracellular injection of EGTA could also suppress the acceleration of the response to bright flashes.

Using an infrared television camera to observe the preparation, we impaled dark-adapted photoreceptors with a micropipette filled with KCl. A 10-μm-diam light spot was then focused onto the most sensitive region of the photoreceptor. After further dark adaptation, the cell was impaled with a second micropipette (see Methods), filled with 0.1 M EGTA (pK = 6.3) and 0.03 M Ca(OH)$_2$ at pH 7. The ratio of 0.3 Ca to EGTA was chosen to maximize the buffering capacity of the EGTA and so to minimize the light-induced changes in pCa. The free calcium concentration in the solution was 0.1 μM, as measured with a calcium-selective macroelectrode.

The photoreceptor was then voltage-clamped to its resting potential and the responses to two flashes, delivering 1,000 and 10,000 effective photons, were recorded (Fig. 6A). The photoreceptor was dark-adapted for 10 min between flashes. A log-log plot of the response per effective photon (Fig. 6C) demonstrates the acceleration (shift to the left) and saturation (reduction in the peak response per photon) that accompanied these flashes. The log-log plot also has a very steep rising slope (cf. Fig. 4C) caused by the abruptness of the rising edge of the responses.

10–100 pl of the CaEGTA solution was then pressure-injected into the photoreceptor (see Methods) and the stimuli were repeated. The cell of Fig. 6 shows that responses recorded after CaEGTA injection were slower and of reduced amplitude compared with those before (Lisman and Brown, 1975b). The total charge transferred during the responses to 1,000 effective photons (the area under the response) was reduced to an average of 30% of that before EGTA injection (seven cells; range, 21–47%). The log-log plots of the responses per effective photon (Fig. 6D) coincided, which demonstrates that, after injection, the responses rose in linear proportion to the stimulus. Moreover, like the responses in the presence of backgrounds (Fig. 4D), the responses initially rose as the fifth power of the time after the flash.

In order to investigate the role of the injected calcium load in determining the response kinetics, we injected cells with 0.1 M EGTA and 0.08 M Ca(OH)$_2$ at pH 7.0. The free calcium concentration in this solution was 1 μM. The protocol for stimulation and injection was the same as for cells injected with 0.3 CaEGTA
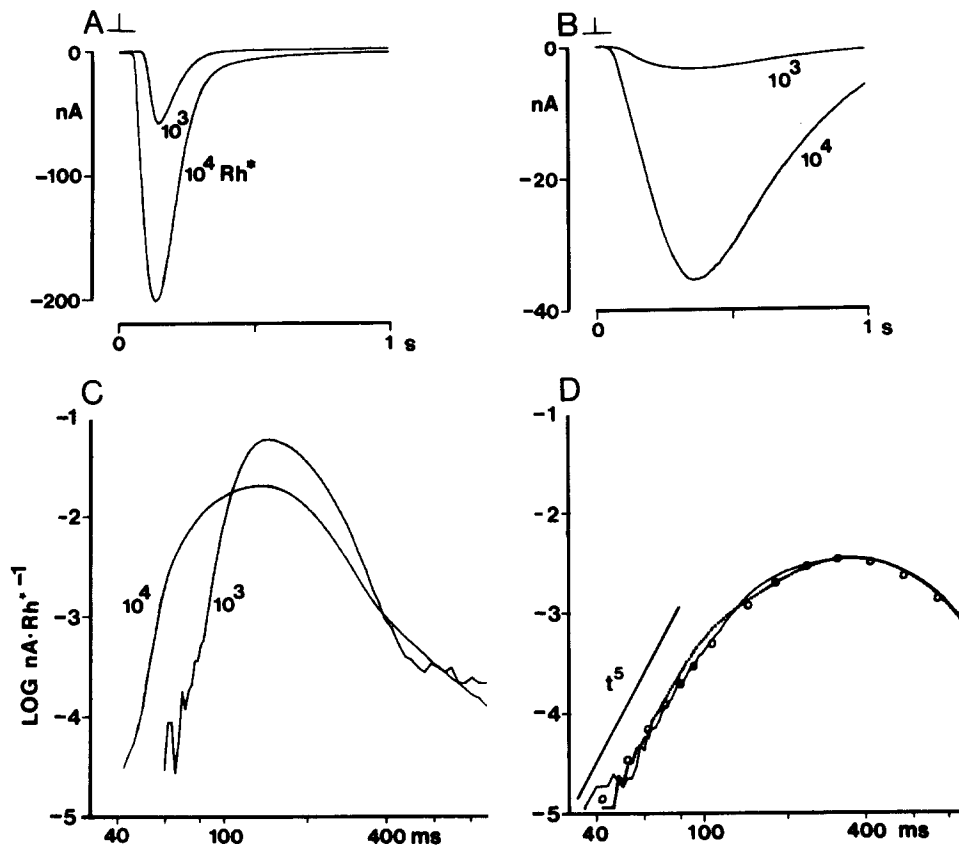

FIGURE 6. (A) Control, dark-adapted responses to flashes delivering 1,000 and 10^4 effective photons to a 5-μm-diam spot centered on the most sensitive region of the photoreceptor. (B) Responses to the same intensity flashes as used in A, recorded after pressure injection of 10–100 pl of a solution containing 0.03 M Ca(OH)$_2$ and 0.1 M EGTA neutralized with KOH. Note the change in the vertical scale, as compared with that of A. (C) The responses in A are divided by the number of effective photons in each flash and are plotted on log-log coordinates. (D) The responses in B are divided by the number of effective photons in each flash and plotted on log-log coordinates. The open circles are the solution to Eq. 14 using n = 6, α = 23 s^{-1}, β = 0.11, and sC = 3.6 × 10^4 nA·photon^{-1}·s^{-5}.

(see above). Eight cells injected with 0.8 CaEGTA responded to flashes with significantly shorter latencies, times to peak, and reduced response areas when compared with seven cells injected with 0.3 CaEGTA (see Table II). Increasing the calcium load increased the initial response by reducing the latency of the

response. The response area was diminished at the higher calcium load because of a more rapid decay of the photocurrent. An example of such an experiment is illustrated in Fig. 7, for comparison with Fig. 6. Just as after injection with 0.3 CaEGTA, cells injected with 0.8 CaEGTA exhibited linear responses to flashes delivering 1,000 and 10^4 effective photons. The initial responses after, but not before, injection rose as the fifth or sixth power of the time after a flash (Fig. 7, C and D).

To quantify the response kinetics after injection, we fitted the responses with the output of a cascade of first-order reactions (see Theory) having $n - 1$ cascaded intermediates that decayed with rate α and one that decayed with rate $\alpha\beta$. The response of this cascade to a flash is given by Eq. 3.

For currents below saturation, Eqs. 3 and 13 predict a response given by

$$i_L = sCN(\alpha - \alpha\beta)^{-(n-1)} \left[e^{-\alpha\beta t} - e^{-\alpha t} \sum_{r=0}^{n-2} \frac{(\alpha - \alpha\beta)^r t^r}{r!} \right], \tag{14}$$

where $s = i_{sat}/\sigma$ is the sensitivity of the current to the transmitter, y.

TABLE II

Characteristics of Responses to 1,000 Rh* After EGTA Injection

CaEGTA	Time to peak	Latency	Area
	ms	*ms*	*nA·s*
0.3	340±98	51±9.6	1.7±0.6
0.8	203±85	39±5	0.89±0.6

Latency is defined as the time taken for the response to a flash delivering 1,000 effective photons to reach 0.03 nA. Values are given as means ± SD.

Of the cells injected with 0.3 CaEGTA, four out of seven were best fitted with $n = 6$, and the remainder with $n = 7$. Of the cells injected with 0.8 CaEGTA, one out of eight was best fitted with $n = 6$, and the remainder with $n = 7$. The response of cells injected with the higher calcium load tended to require higher values of α and sC in order to obtain satisfactory fits when compared with responses of cells injected with the lower calcium load, which led to the shorter times to peak and latencies of Table II. β was not significantly altered by the calcium load, and ranged from 0.09 to 0.15 when responses were fitted after injection of 0.8 CaEGTA and from 0.06 to 0.12 when responses were fitted to 0.3 CaEGTA. Examples of fits of Eq. 14 to the data are shown in Figs. 6D and 7D by the open circles.

We conclude that increasing the injected calcium concentration shortens the response time scale and reduces the total charge transferred during the response, but it does not alter the basic shape of the response or its linearity when bright flashes are delivered after injection of CaEGTA. After injection, responses follow kinetics similar to those of vertebrate cones, to which Eq. 3 has also been applied using $n = 6$ or 7 (Reaction Scheme I of Theory; Baylor et al., 1974).

Control Injections

Control injections of 100 mM K aspartate with 10 mM HEPES, pH 7, reduced the amplitude of the response to the same stimulus protocol as was used above.
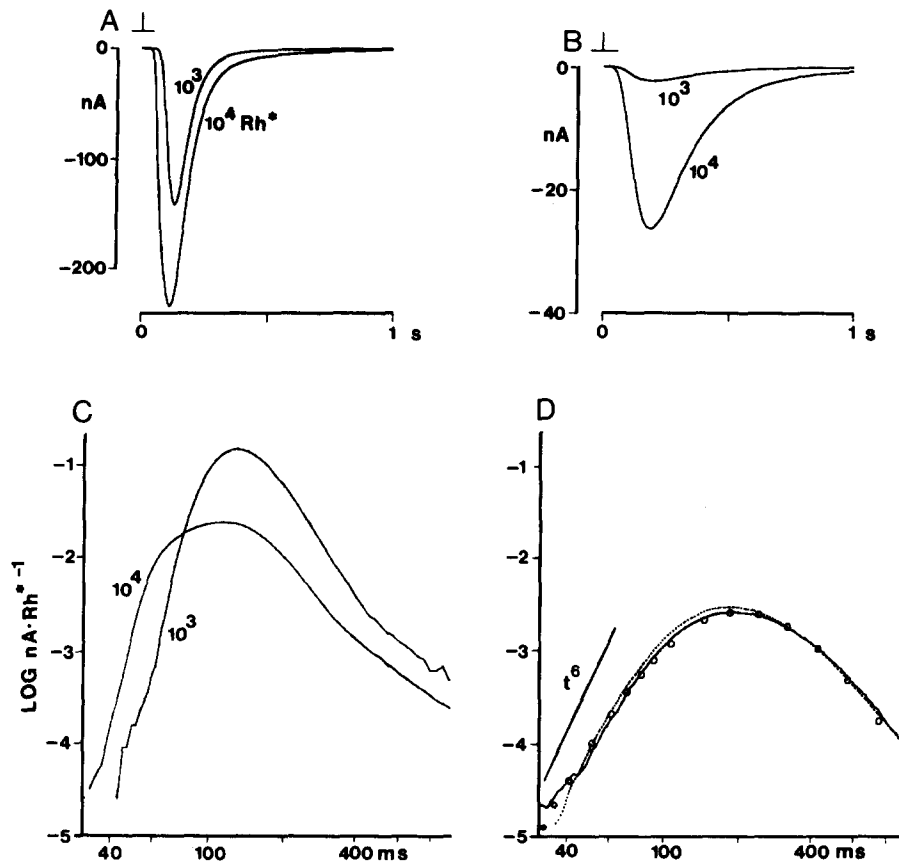

FIGURE 7. (A) Control, dark-adapted responses to flashes delivering 1,000 and 10^4 effective photons to a 5-μm-diam spot centered on the most sensitive region of the photoreceptor. (B) Responses to the same intensity flashes as used in A, recorded after pressure injection of 10–100 pl of a solution containing 0.08 M $Ca(OH)_2$ and 0.1 M EGTA neutralized with KOH. Note the change in the vertical scale, as compared with that of A. (C) The responses in A are divided by the number of effective photons in each flash and are plotted on log-log coordinates. (D) The responses in B are divided by the number of effective photons in each flash and plotted on log-log coordinates. The open circles are the solution to Eq. 14 using n = 7, $\alpha = 42$ s^{-1}, $\beta = 0.13$, and $sC = 2.49 \times 10^7$ nA·photon^{-1}·s^{-6}.

The charge transferred during the response to 1,000 Rh* was reduced to between 27 and 67% (mean, 50%; five cells) of that before injection. However, in contrast to CaEGTA injection, no change in the time course or initial acceleration of the response was noted after injection of aspartate. Injection of

0.1 M K HEPES, pH 7.0, also failed to alter the time course or initial acceleration of the response, although the charge transferred during the response to 1,000 Rh* was reduced to between 28 and 82% of that before injection (mean, 56%; six cells). These control experiments indicate that a considerable fraction of the 70% mean reduction in the total charge transferred during the response after injection of 0.3 CaEGTA could be an artifact of the injection method, and that artifactual reductions in the transferred charge are not associated with changes in the acceleration of the response to bright flashes or in the response kinetics.

Wavelength Dependence and Reversal Potential of the Response to Bright Flashes

The experiments described in previous sections used white light. We illuminated photoreceptors with monochromatic light at two different wavelengths, delivering equal numbers of effective photons. Photoreceptors were dark-adapted and dim flashes of diffuse 460 nm light were delivered, each producing ~10 discrete waves. The intensity of a beam of 601 nm diffuse light was adjusted so as to elicit approximately the same number of discrete waves per flash. The responses to the flashes were then recorded and the intensity of both beams was increased in steps of several log units, allowing time for dark adaptation between presentations. As in the experiments of Figs. 1 and 3, diffuse flashes delivering 1,000 or more effective photons accelerated the rising edge of the response. The waveforms and latency of the responses to 460 and 601 nm light are similar for flashes delivering up to 3×10^5 effective photons. We can therefore provide no evidence that the waveform of the response to bright flashes is controlled by a visual pigment with a spectral sensitivity at 460 and 601 nm different from that generating the response to dim flashes.

It is possible that the increase in the initial response to bright flashes arises from the activation of a novel ionic conductance that displays a short latent period. We therefore decided to check whether the initial photocurrent produced during the exposure to a bright flash reversed at the same membrane potential as the later photocurrent.

For four cells investigated, the response to a bright flash recorded at 75 ms reversed between +7 and +27 mV, as did the response recorded at 140 ms. We never observed biphasic responses at any holding potential. This was previously observed by Millecchia and Mauro (1969b). Our data are essentially the same as those shown in their Fig. 5 and for that reason we do not illustrate it here. We can therefore provide no evidence to suggest that the shorter latency of the response to a bright flash is due to a novel ionic conductance with a reversal potential different from that of the later response.

DISCUSSION

Initiation of Nonlinearity

The first quantitative studies of invertebrate photoreceptors recognized the nonlinear nature of the response to a bright flash. To account for this, Fuortes and Hodgkin (1964) and Borsellino et al. (1965) proposed that bright light accelerates the rate of decay of intermediates in the visual cascade. The reactions of the visual cascade mediating the response to a dim flash were presumed to

behave linearly. Model responses to a bright flash initially followed the linear estimate but fell below it at later times. In contrast to these earlier studies on *Limulus* lateral eye, later studies using *Limulus* ventral eyes and fly retinular cells found that responses delivering >100 effective photons initially exceeded the linear estimate (Fein and Charlton, 1977*b*; Brown and Coles, 1979; Hamdorf and Kirschfeld, 1980; Dubs, 1981). This suggests that bright flashes accelerate not only the rate of decay of visual intermediates, but also their rate of production.

The present paper confirms the observation that for a fixed state of adaptation, bright flashes reduce the latency of the response (Millecchia and Mauro, 1969*a*). As a result, the response of the dark-adapted receptor initially rises as the fourth power of light intensity when >100 effective photons are delivered to ~100 μm^2 of the most sensitive region of the cell. In the dark-adapted state, there is no reliably detectable current (i.e., >0.1 nA) for which linear summation is applicable to the initial response to bright flashes. The nonlinear relationship between photocurrent and intensity must therefore be initiated before, or simultaneously with, the generation of significant photocurrent. We can provide no evidence that the accelerated initial response to bright flashes is mediated by an ionic conductance with a reversal potential significantly different from that mediating the response at later times. Neither can we provide evidence that the acceleration is mediated by a visual pigment with an absorption at 460 and 601 nm different from that of rhodopsin. We therefore propose that the acceleration of the initial dark-adapted response to bright flashes is initiated by rhodopsin and that it involves a modification to the kinetics of the same ionic channels that mediate the response to dim flashes.

A Model for the Initial Response

Our observation of an initial "supralinearity" of responses to bright flashes necessarily implies the cooperative action of more than one particle released by a photoisomerization. In the theoretical section of this paper, we describe how the initial deviation from linearity could result if, in addition to initiating a cascade that results in the production of internal transmitter particles, light also initiated the release, via a parallel cascade, of an agent that accelerates the rates of production of intermediates in the first cascade. The release of the accelerating agent is assumed to be localized to the region of a photoisomerization. As a result, the response to a spot of light rises faster than that to a diffuse light delivering the same total number of effective photons to the cell (Fig. 3).

The nonlinear combination of the two cascades results in dark-adapted responses to bright flashes that rise as the 17th power of the time after a flash and as the 4th power of flash intensity. If the background illumination is assumed to increase the background concentration of the accelerating agent, then the reduced latency and the initially linear response of the light-adapted photoreceptor can be explained.

Calcium as the Accelerating Agent

If the release of accelerating agent in our model is suppressed, a drastic modification of the dark-adapted response occurs. The rates of reactions in the cascade

producing transmitter paticles are then slowed to rates determined by the background concentration of accelerating agent. Nonlinear acceleration of the initial response with increasing flash intensity is abolished. In the model of Eq. 9, similar changes can be obtained by setting B to zero. The responses of the model under these conditions will initially rise as the fifth power of the time after a flash. We have found that intracellular injection of EGTA results in slow, linear responses to bright flashes that rise as the fifth and sixth power of the time after the flash and can be modeled as a linear cascade of first-order reactions having six or seven intermediates. We regard this result as evidence for the proposal that the accelerating agent is the rise in intracellular calcium that follows a flash. Injection of EGTA suppresses the transient rise in intracellular calcium that follows a bright flash of light (Brown and Blinks, 1974).

Calcium injection accelerates the initial response of Limulus ventral photoreceptors (Brown and Lisman, 1975). Calcium transients after bright flashes rise to a peak ~200 ms after a flash, reaching a maximum of up to 40–100 μM (Brown and Blinks, 1974; Brown et al., 1977; Levy and Fein, 1985). The rise in calcium therefore may be fast enough to mediate an acceleration of the initial response of the receptor. Furthermore, the steady state level of the intracellular free calcium concentration is elevated in the light-adapted state (Levy and Fein, 1985). The changes in the initial kinetics of the response can be explained in our model if the resting concentration of accelerating agent is elevated approximately fivefold. Several qualitative observations of the calcium signal after bright flashes and during light adaptation are therefore compatible with our proposal. However, no study has so far established the exact timing of the initial release of calcium and the photocurrent.

Calcium diffuses slowly inside cells with a diffusion constant of ~10^{-7} cm$^2 \cdot$s^{-1} (Kushmerick and Podolsky, 1969; Gorman and Thomas, 1980; also see Fein and Charlton, 1978). The limited diffusion of calcium could account for the localization of the accelerating agent that is necessary to our model.

Martinez and Srebro (1976) and Lisman (1976) observed that lowering extracellular calcium increased the latencies of quantal events. They proposed that the reaction of intermediates in the visual cascade with calcium may be a necessary step in the activation of the receptor. Our model makes the additional proposal that some of the calcium comes from that released by light as well as from the resting intracellular concentration. Bolsover and Brown (1985) have observed that, under conditions of low extracellular and intracellular calcium, the responses of ventral photoreceptors increased more than linearly with increasing flash intensity. They proposed that this behavior arises from a requirement in excitation for calcium, which was released by bright flashes. Under these conditions, injections of calcium increase sensitivity to light. We suggest that the calcium requirement that they observed may simply be an accentuation of the phenomena reported in this paper at normal calcium concentrations.

Prolonged iontophoretic injection of calcium is known to desensitize the response of the cell by several log units (Brown and Lisman, 1975). A wave of desensitization must therefore follow the initial acceleration of the response by calcium. Certainly, bright flashes that evoke acceleration of the initial response desensitize the cell subsequently. Fein and Charlton (1977b) also noted that the

"enhancement" of the initial response to test flashes superimposed upon bright steps is followed by adaptation if the test flash follows the onset of the bright step by >200 ms. Calcium release therefore appears to play a dual role, important to both excitation and adaptation of the response.

Responses to Dim Flashes

Our assumption that the release of the accelerating agent is localized means that, for dim flashes, the release of the agent could significantly modify the kinetics of the response to a given quantum without affecting the responses to neighboring quanta. Our model predicts that the local effect of the accelerating agent results in average responses to dim flashes that rise abruptly, as high powers of the time after the flash, but which summate linearly with increasing light intensity. The overlap of agent released from neighboring isomerizations is insignificant until more than one photon is effective for every square micron of cell surface, when nonlinear summation begins. We have no direct evidence, however, that our model applies to the response to single quanta, except for the observation that intracellular injection of EGTA slows the response to dim flashes just as it slows the response to bright flashes. We cannot unequivocally determine whether the accelerating agent acts on the "latency process" or the "bump process" that underlies single photon events (Wong et al., 1982). Nor can we determine whether the nonlinear step in transduction occurs at or before the gating of the ionic channels.

The results of this paper suggest that release of calcium by light accelerates the initial events of transduction. Calcium release by light is neither sufficient nor necessary for the generation of photocurrent by light in our model. This is in accord with the inability of calcium to excite the cell directly when ionophoretically injected at sufficient concentration to accelerate the initial response to a flash (Brown and Lisman, 1975). Also, our model is in accord with the inability of EGTA to abolish the response to light. We cannot, however, rule out the possibility that higher concentrations of calcium than those imposed in the experiments of Brown and Lisman (1975) are able to directly excite the cell. Recently, Fein et al. (1984) and Brown et al. (1984) have demonstrated photoreceptor excitation by inositol 1,4,5-trisphosphate ($InsP_3$), an agent that rapidly causes a rise in intracellular calcium in *Limulus* ventral photoreceptors (Corson et al., 1984; Brown and Rubin, 1984). The release of calcium by rapid light-induced breakdown of polyphosphoinositide may be the biochemical basis of the synergistic role of calcium in excitation that we propose in this paper.

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