The Role of Metarhodopsin in the Generation of Spontaneous Quantum Bumps in Ultraviolet Receptors of *Limulus* Median Eye

Evidence for Reverse Reactions into an Active State

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ABSTRACT The origin of spontaneous quantum bumps has been examined in the ultraviolet photoreceptors of *Limulus* median eye. These cells have a rhodopsin with a λ_{max} at 360 nm and a stable photoproduct, metarhodopsin, with a λ_{max} at 470 nm. The steady state rate of spontaneous quantum bumps was found to be higher when the metarhodopsin concentration was high than when the rhodopsin concentration was high. This result implicates metarhodopsin in the generation of spontaneous quantum bumps. Furthermore, this result is consistent with the idea that the reaction which inactivates metarhodopsin (terminates the ability of metarhodopsin to initiate the reactions leading to a quantum bump) is reversible and that such reversions can be a significant source of spontaneous quantum bumps. Given that the rate of spontaneous quantum bumps is ~1/s under conditions where the number of inactive metarhodopsin molecules is $\sim 10^9$, it follows that the molecular switch that inactivates metarhodopsin reverses with a probability of $<10^{-9}$. A model is presented of how a molecular switch with this reliability might be constructed.

INTRODUCTION

Both vertebrate rods and many types of invertebrate photoreceptors generate observable responses to single photons (Fuortes and Yeandle, 1964; Baylor et al., 1979). The frequency of such responses increases with light intensity, but similar events also occur in complete darkness (Adolph, 1964; Baylor et al., 1980). The origin of these spontaneous events remains unclear despite our knowledge of many factors that can alter their rate: in both invertebrate and vertebrate photoreceptors, the rate rises steeply with temperature (Srebro and Behbehani, 1972; Baylor et al., 1980) and is transiently elevated during the recovery period after bright illumination (Fein and Hanani, 1978; Lamb, 1980). Recent work in *Limulus* photoreceptors indicates that the frequency of spontaneous events can also be increased by a variety of pharmacological agents (Fein and Corson, 1981; Bolsover and Brown, 1982) and that it can be reduced by high-energy nucleotides (Stern et al., 1985) or by synaptic input into the photoreceptor (Kaplan and Barlow, 1980).

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The diagram in Fig. 1 schematizes the early reactions in visual transduction and provides a useful framework for discussing the relationship between spontaneous and light-induced quantum bumps. Light triggers the conversion of rhodopsin to a thermally stable but inactive form of the photoproduct, metarhodopsin. During the transition between these states, the visual pigment passes transiently through an active state that turns on the amplification cascade (Cone, 1973; Bacigalupo and Lisman, 1983) underlying the single photon response. Although the transition kinetics of invertebrate visual pigments have not been

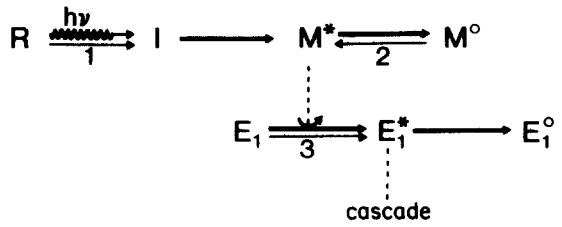

FIGURE 1. Possible reactions that could generate spontaneous quantum bumps. In reaction 1, rhodopsin (R) spontaneously isomerizes, giving rise to active metarhodopsin (M*). In reaction 2, inactive metarhodopsin (M°) spontaneously reverts to M*. In reaction 3, an enzyme (E) that is part of the amplification cascade of transduction turns on spontaneously. The transduction cascade during a photon-induced quantum bump works as follows: the photon converts rhodopsin to an intermediate(s) (I) that decays to M*. Before being inactivated to M°, M* converts multiple E_1's to their active state E_1^*. Before being inactivated to E_1°, each E_1^* activates molecules of the next stage in the cascade, and so on. In the last stage of the cascade, channels are transiently activated, leading to generation of the receptor potential (quantum bump). The conversion of metarhodopsin to rhodopsin by light occurs but is not shown. Insofar as metarhodopsin is described here as a thermally stable state, the scheme applies only to *Limulus* photoreceptors and other invertebrates with a stable metarhodopsin (Hillman et al., 1983).

studied extensively, the available evidence suggests that metarhodopsin is formed very rapidly after a flash (Kirschfield et al., 1978; also see Lisman and Sheline, 1976). Thus, the active state of the visual pigment is likely to be a form of metarhodopsin, and we tentatively label it M*. Quantum bumps like those produced by light, but occurring spontaneously, could occur in several ways. One type of reaction that could generate events in the dark is the thermal isomerization of rhodopsin (reaction 1 in Fig. 1), i.e., a spontaneous transition from R to M* (Srebro and Behbehani, 1972). A second possibility is that the reaction which inactivates M* is reversible (reaction 2); thus, M* may be created from M° (Lamb, 1981). This reaction can be of only transient significance in vertebrate photoreceptors since metarhodopsin is unstable in vertebrates. In most invertebrates, however, the reaction could contribute to the steady state properties of the cell since inactive metarhodopsin is thermally stable and can exist in high concentration (see Hillman et al., 1983). A third possibility is that spontaneous bumps are due to spontaneous activation of a secondary process in

the amplification cascade (reaction 3 in Fig. 1) and are thus not related to events in either rhodopsin or metarhodopsin (Corson and Fein, 1983).

The reactions illustrated in Fig. 1 may all occur, but it would be of interest to determine which reaction is the primary source of spontaneous events under given conditions. In order to explore this question, I have used the ultraviolet (UV) photoreceptors of *Limulus* median eye. These cells have a rhodopsin with a λ_{max} at 360 nm (Nolte and Brown, 1969) and a stable metarhodopsin with a λ_{max} at 470 nm (as shown in this paper). Thus, rhodopsin and metarhodopsin can be interconverted by chromatic illumination to produce a cell that contains primarily rhodopsin or primarily metarhodopsin. If the spontaneous isomerization of rhodopsin (reaction 1) is the primary source of spontaneous quantum bumps, the spontaneous rate should be highest when the rhodopsin concentration is highest. Conversely, if reversion of inactive metarhodopsin to active metarhodopsin (reaction 2) is the primary source of spontaneous quantum bumps, the rate should be highest when the metarhodopsin concentration is highest. Finally, if spontaneous quantum bumps are due to reactions secondary to the visual pigment (reaction 3), the rate should be independent of the rhodopsin-to-metarhodopsin ratio. The results presented here show that the spontaneous quantum bump rate is highest when cells primarily contain metarhodopsin and therefore support the idea that the inactivation of metarhodopsin is reversible and that such reverse reactions can be a significant source of spontaneous quantum bumps when the concentration of metarhodopsin is high. Preliminary accounts of this work have been presented elsewhere (Lisman et al., 1983; Lisman, 1984).

METHODS

Median eyes were removed from the cornea and pinned into the recording chamber with the corneal side up. After treatment with pronase (20 mg/ml for 1 min), cells were bathed in artificial seawater containing 10^{-6} M tetrodotoxin. This toxin was added in order to simplify the detection of quantum bumps by eliminating the small action potentials recorded from these cells (Nolte and Brown, 1972*a*). Intracellular recordings were made with conventional microelectrodes that were filled with 3 M KCl and had resistances of 10–40 MΩ. Cells were penetrated from the corneal side. UV cells were distinguishable from visible cells (Nolte and Brown, 1969) by their inability to respond to orange light.

The optical system was a modification of that described by Lisman and Bering (1977). Early receptor potentials (ERPs) were evoked by flashes from a photographic strobe light. A shuttered quartz-iodide light source was used for chromatic adaptation. For photoregenerating rhodopsin from metarhodopsin, light was passed through an orange filter (OG550; Schott Optical Glass, Inc., Duryea, PA). For converting rhodopsin to metarhodopsin, a UV filter (UG11; Schott Optical Glass, Inc.) was used.

The action spectrum of metarhodopsin was measured using the following procedure. Flashes were filtered with narrow-band (10-nm bandwidth) interference filters. 8 or 16 ERPs at each wavelength were averaged using a signal averager. The cell was continuously irradiated with a UV background light to ensure that the cell contained the maximal concentration of metarhodopsin when each flash was given. For wavelengths near the λ_{max} of metarhodopsin, it was necessary to attenuate the flashes slightly with neutral density filters to ensure that the ERPs were in the linear range of the response-intensity curve.

The relative energy of the stimulus at each wavelength was measured with a calibrated photodiode and the data were plotted (Fig. 3) as the relative response per incident photon. The procedure for measuring the spectrum of rhodopsin was similar, except that the cell was continuously irradiated with orange light to ensure that it contained a maximal concentration of rhodopsin at the onset of each flash.

To estimate the number of rhodopsin molecules in a cell or group of cells using the method of Lisman and Bering (1977) (see p. 177), it was necessary to know I_2, the intensity that half-saturates the rhodopsin ERP, and I_1, the flash intensity that evokes an average of one bump per flash. The maximum flash intensity (I_0) of the strobe unit was sufficient to saturate the metarhodopsin ERP, but insufficient to saturate the rhodopsin ERP. Therefore, to determine I_2 I had to use the following indirect strategy. Starting with a maximal concentration of rhodopsin, a white flash of intensity I_0 was given. The metarhodopsin created by this flash was then exposed to a yellow flash (GG495; Schott Optical Glass, Inc.), resulting in an ERP of amplitude V_m. This was then compared with the maximum metarhodopsin ERP ($\bar{V}_m$) evoked by a yellow flash (after exhaustive UV irradiation). I_2 was calculated according to the formula:

$$I_2 = (I_0)(0.5\ \bar{V}_m)/V_m. \quad (1)$$

To measure the intensity evoking a bump, I_1, the flash intensity was greatly attenuated using calibrated neutral density filters until the probability of any response to the flash was ~0.5. 50 flashes of this intensity were given, from which the probability of no response [$P(0)$] was computed. The average number of bumps per flash was computed from $P(0)$ using the Poisson equation. I_1 could then be calculated using the linear relationship between the average number of bumps per flash and light intensity. In these experiments, the probability of spontaneous bumps was so low (<0.3/s) that it could be ignored. There was some uncertainty about whether small baseline deflections were very small quantum bumps or merely random fluctuations. Therefore, in this and other experiments involving the counting of quantum bumps, I adopted the convention that to be counted as a bump a deflection had to have the time course of a bump and had to have an amplitude at least 1.5 times the peak-to-peak baseline noise.

RESULTS

Before proceeding with the main goal of this paper, it was first necessary to examine some of the basic properties of the UV pigment in median photoreceptors. This was done using the ERP as a measure of the visual pigment. The ERP is a small voltage change that precedes the large depolarizing response to light. The ERP is produced by a light-induced dipole shift within the visual pigment molecule itself and is observable only in response to bright flashes. The polarity of the ERP is determined by the state of the visual pigment molecules and the amplitude is proportional to the number of pigment molecules present (for a review of the ERP, see Cone and Pak, 1971).

λ_{max} *of the Metarhodopsin in UV Cells*

Fig. 2 illustrates intracellular recordings of the ERP in UV cells of the *Limulus* median eye and shows that the polarity of the ERP evoked by a flash of white light depended on the wavelength of the previous illumination. If the cell was preirradiated with orange light, the ERP was negative-going (Fig. 2*a*). If the cell was preirradiated with UV light, the ERP was positive-going (Fig. 2*b*). The ERP was once again negative (Fig. 2*c*) after a subsequent orange irradiation. The

spectrum for the positive ERP is shown in Fig. 3. It has a peak in the blue-green and can be fit by a nomogram with a λ_{max} at 470 nm. Because of the low energy output of our flash lamp in the UV, it was not possible to obtain data about the negative ERP at wavelengths below 360 nm. Nevertheless, from the data available (Fig. 3), it is clear that the negative ERP is generated by pigment with a λ_{max} in the UV. The spectrum with a λ_{max} at 360 nm in Fig. 3 is that of the late receptor

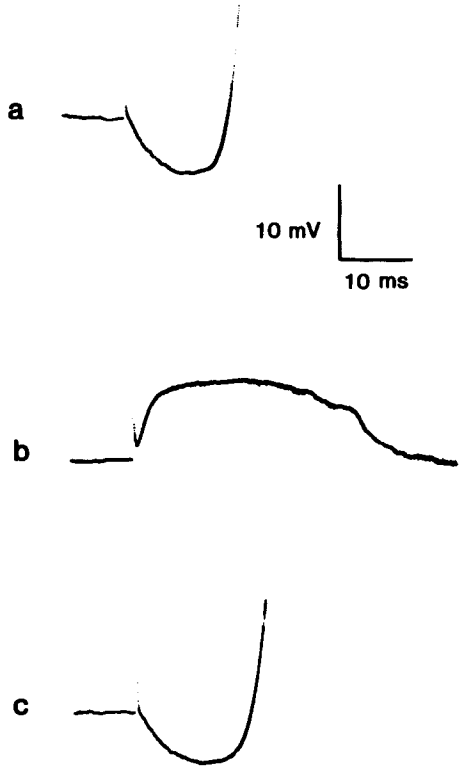

FIGURE 2. Effect of the wavelength of preadaptation on the polarity of the ERP evoked by a white flash. (*a*) After exhaustive preadaptation with orange light. (*b*) After exhaustive preadaptation with UV light. (*c*) A repetition of *a*. In *a* and *c*, the late positive-going component is the late receptor potential. This is not present in *c* because preadaptation with UV light causes a maximal long-lasting activation of the late receptor potential (see Fig. 4*b*). A small electrical artifact is evident at the beginning of each ERP. The flash duration was ~1 ms.

potential (Nolte and Brown, 1969); the three measured points for the negative ERP fall nicely on this curve. It can be concluded that UV cells contain a rhodopsin with λ_{max} near 360 nm and a metarhodopsin with λ_{max} near 470 nm, wavelengths similar to those of other UV visual pigments (Hamdorf, 1979). The polarities of the rhodopsin and metarhodopsin ERPs, respectively, are consistent with those in squid (Hagins and McGaughy, 1967), barnacle (Minke et al., 1973), and *Limulus* ventral photoreceptors (Lisman and Sheline, 1976).

Absence of Dark Regeneration

Experiments of the kind shown in Fig. 4 were designed to determine whether metarhodopsin is thermally stable or whether it regenerates to rhodopsin in the dark. Trace 4 in Fig. 4*a* shows a positive ERP measured 1 h after conversion of rhodopsin to metarhodopsin, and trace 2 shows an ERP in the same cell measured 6 h after a similar conversion (see Fig. 4*b*). If rhodopsin had regenerated during this period, the ERP would have become negative. Because trace 2 was positive and had nearly the same amplitude as trace 4, it can be concluded that there was little conversion of metarhodopsin to rhodopsin in the dark.

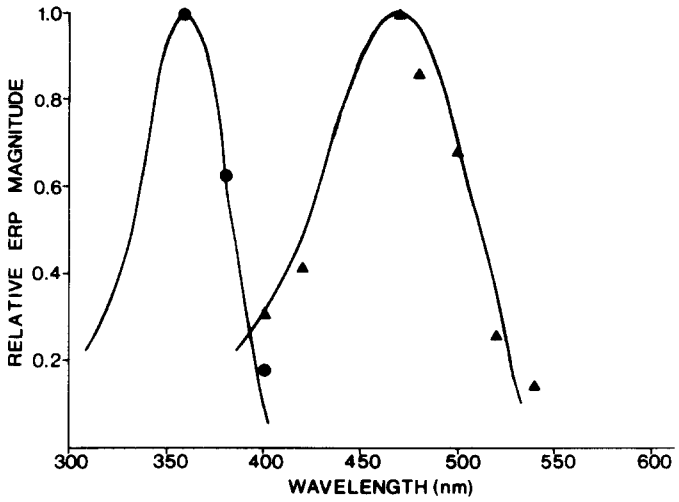

FIGURE 3. Action spectrum of the positive (metarhodopsin) ERP (▲) and the negative (rhodopsin) ERP (●). The procedure for obtaining these spectra is described in Methods. The solid lines were constructed using an iodopsin nomogram provided by P. K. Brown. In the case of the metarhodopsin nomogram (right), a reasonable fit to the data was obtained for a nomogram with λ_{max} at 470 nm. The λ_{max} of the rhodopsin nomogram (left) was taken to be the λ_{max} of the late receptor potential (360 nm) (Nolte and Brown, 1969).

In the course of the experiments described above, it was noted that the metarhodopsin ERP must have been slightly influenced by some other factor. In *Limulus* UV cells, as in most invertebrate photoreceptors, light that converts a large fraction of rhodopsin to metarhodopsin produces a long afterpotential that decays in ~1 h (Nolte and Brown, 1972; see Fig. 4*b*). The metarhodopsin ERP measured at the beginning of the afterpotential (traces 1 and 3 in Fig. 4*a*) was somewhat different in waveform from the metarhodopsin ERP measured toward the end of the afterpotential (traces 2 and 4). This may indicate that the ERP waveform is dependent on the absolute membrane potential; in *Limulus* ventral photoreceptors, the ERP generated by metarhodopsin decreases as the membrane voltage is made more positive (J. Lisman, unpublished observation). Alternatively, these changes in the ERP may reflect changes in visual pigment

molecules that correlate with the decline of the afterpotential. Further work will be needed to clarify this point.

Number of Rhodopsin Molecules

By using Eq. 2 (Lisman and Bering, 1977), the number of rhodopsin molecules in a cell (N) can be estimated electrophysiologically from the ratio of I_2 to I_1,

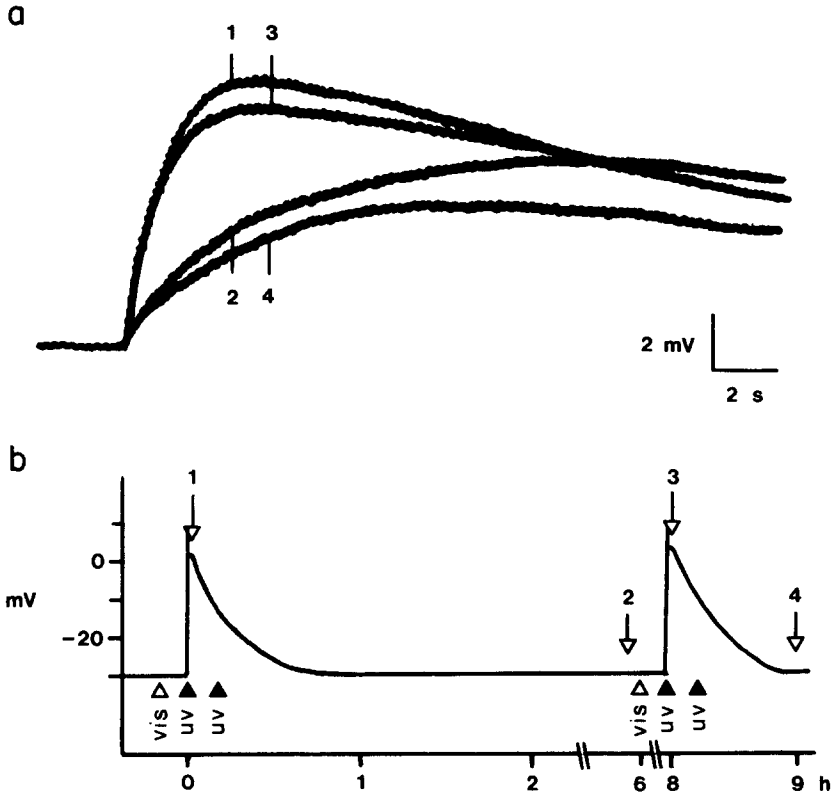

FIGURE 4. Absence of regeneration of rhodopsin from metarhodopsin in the dark. (*a*) Superimposed metarhodopsin ERPs evoked by yellow flashes. Response 4 was evoked 6 h after maximal conversion of rhodopsin to metarhodopsin and has nearly the same amplitude and waveform as response 2, which was evoked only 1 h after similar irradiation. Thus, there was little or no dark regeneration between 1 and 6 h. Responses 1 and 3 were generated by metarhodopsin and were evoked within minutes after conversion of rhodopsin to metarhodopsin. Possible reasons for the differences in waveform between these responses, which were superimposed on a depolarizing afterpotential (see *b*), and responses 2 and 4, which were recorded near resting potential, are discussed in the text. (*b*) A schematic of the membrane potential vs. time indicating the sequence of irradiations and the times at which the ERPs in part *a* were evoked. VIS and UV stand for exhaustive irradiation with orange and UV light, respectively. The purpose of the UV stimuli given just after flashes 1 and 3 was to reconvert to metarhodopsin any rhodopsin that was generated by flashes 1 and 3.

where I_2 is the relative flash intensity that half-saturates the ERP and I_1 is the relative flash intensity that evokes an average of one quantum bump:

$$N = 2.9 \frac{I_2}{I_1}. \quad (2)$$

N was measured (see Methods) in four experiments under conditions in which most of the visual pigment was in the rhodopsin state. The average number of pigment molecules was $\sim 2 \times 10^9$ (Table I). There are some indications that UV cells in the median eye are coupled electrically (Nolte and Brown, 1972*a*, *b*). If this was the case in the experiment reported in Table I, many of the recorded quantum bumps may not have originated in the impaled cell, and it would be more correct to think of N as the number of rhodopsin molecules in a group of coupled cells. In any case, it can be safely concluded that the spontaneous quantal responses described in the next section are generated within a cell or a pool of coupled cells that contains on the order of 10^9 visual pigment molecules.

TABLE I

Estimate of Number of Rhodopsin Molecules

Cell	$\bar{V}_m$	Number of molecules (N)
	mV	
1	8	5.2×10^8
2	13	2.9×10^8
3	13	6.5×10^9
4	6	1.5×10^9
		Average: 2.2×10^9

Effect of Metarhodopsin Concentration on Spontaneous Bump Rate

In the experiments described in this section, the preparation was first irradiated with UV light to generate a maximal concentration of metarhodopsin. The rate of spontaneous quantum bumps was measured after it reached a stable level. The cell was then irradiated with orange light to create a maximal concentration of rhodopsin. The rate of spontaneous bumps in this high-rhodopsin condition was measured and compared with that in the high-metarhodopsin condition. The major experimental difficulty was that it took many hours for the rate of spontaneous quantum bumps to become stable after conversion of rhodopsin to metarhodopsin. Massive conversion of rhodopsin to metarhodopsin produced a long afterpotential. By the end of 1 h, the smooth afterpotential had decayed and spontaneous quantum bumps were observable. The rate of these spontaneous bumps was initially very high (Fig. 5), but then slowly declined over a period of several hours. Only after 4 h did the rate finally reach a steady state (Fig. 5).

Figs. 5 and 6 illustrate the dependence of the steady state spontaneous quantum bump rate on the state of visual pigment. The upper traces of Fig. 6 were taken in the high-metarhodopsin condition 6.5 h after UV irradiation. At this time, the spontaneous quantum bump rate had been stable for the previous 2 h. The middle set of traces was recorded 30 min after rhodopsin was regenerated from

metarhodopsin with orange light. The spontaneous rate in this high-rhodopsin condition was much lower than that in the high-metarhodopsin condition. Experiments of this kind were completed on six cells (Table II). In all cells examined, conversion from the high-metarhodopsin condition to the high-rhodopsin condition produced a dramatic reduction of the spontaneous quantum bump rate. The magnitude of the reduction varied from cell to cell, with the lowest value being 3.6 and the highest 36.7. The reduction in rate occurred with no apparent delay after the conversion of metarhodopsin to rhodopsin.

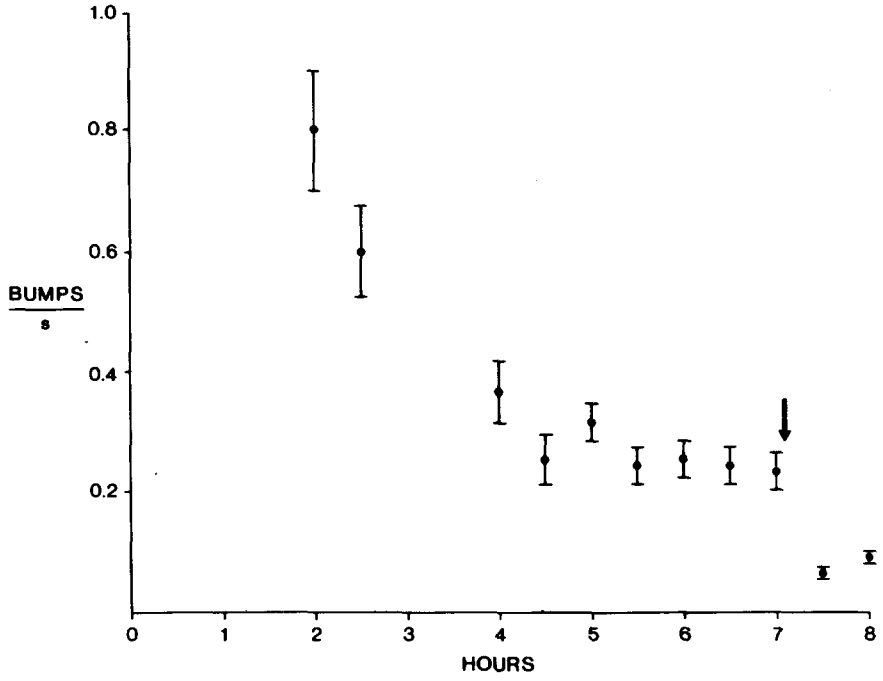

FIGURE 5. Spontaneous quantum bump rate (mean ± SD) as a function of time after conversion of rhodopsin to metarhodopsin (which occurred at $t = 0$) and after conversion of metarhodopsin to rhodopsin (which occurred at the time marked by the arrow). Data are from cell 1 of Table II. During the initial hour, bumps occurred at such a high rate that they fused to form a smooth afterpotential and could not be counted individually.

An interesting feature of spontaneous quantum bumps in *Limulus* ventral photoreceptors is that their average amplitude is smaller than that of light-induced quantum bumps (see Corson and Fein, 1983). To determine if this was also the case in UV cells of the median eye, I measured the amplitude of quantum bumps in light and in darkness. These measurements were made in the high-metarhodopsin condition after a stable quantum bump rate was achieved. From these measurements, the amplitude of light-induced quantum bumps was calculated using the procedure described in Table III. In five of six cells tested, spontaneous bumps were smaller than light-induced ones.

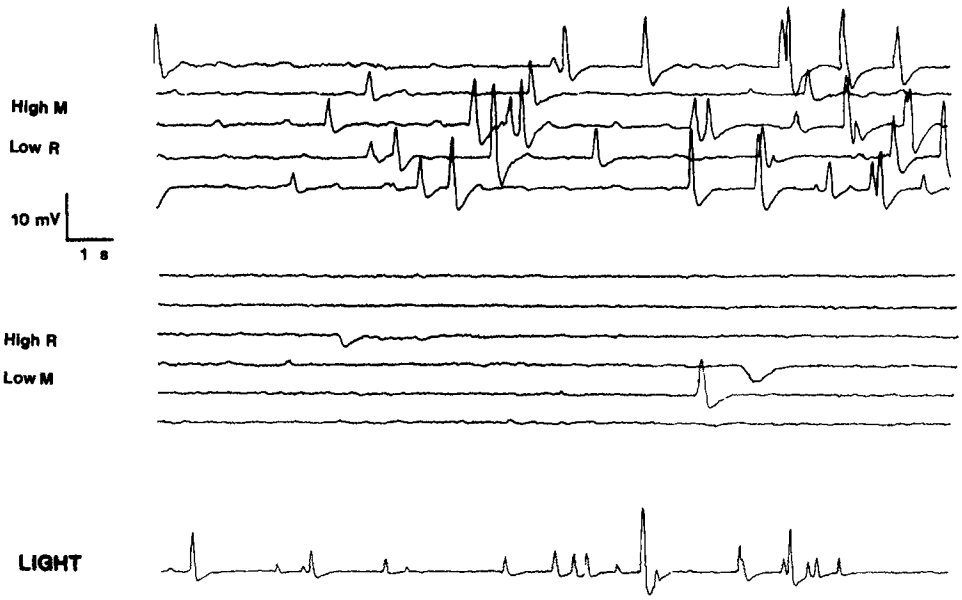

FIGURE 6. Tracings of spontaneous quantum bumps in the high-metarhodopsin condition (after a steady rate was achieved) are shown in the upper traces and in the high-rhodopsin condition in the middle traces. The lowest record (marked "light") illustrates the effect of dim light when the cell was in the high-rhodopsin condition. Thus, even though the cell produced almost no spontaneous bumps (as shown in the middle traces), it was still able to produce large bumps in response to light. The origin of the brief downward deflections in the middle traces is not known. Data are from cell 4 of Table II.

TABLE II

Dependence of Spontaneous Quantum Bump Rate on Relative Concentration of Rhodopsin (R) and Metarhodopsin (M)

	High M			High R		
Cell	Time after R-to-M conversion	Rate (M)	Duration of stability*	Time after M-to-R conversion	Rate (R)	Rate (M) / Rate (R)
	h	*bumps/s*	*h*	*h*	*bumps/s*	
1	7.0	0.24	2.5	1.0	0.06	4.0
2	6.8	0.40	1.0	1.0	0.11	3.6
3	8.0	0.37	1.3	1.3	0.05	7.4
4	5.5	1.10	2.3	2.0	0.03	36.7
5	7.0	3.53	—‡	0.5	0.97	3.9
6	8.0	0.82	—‡	0.5	0.14	5.9

* Duration of bump rate stability in high-M condition before measurement of rate.

‡ Cells were impaled 7–8 h after the R-to-M conversion. The bump rate was then measured within the next hour. Based on cells 1–4, the rate should be stable at these long times after R-to-M conversion.

DISCUSSION

The principal finding reported here is that the steady state rate of spontaneous quantum bumps in cells containing mostly metarhodopsin is higher than in cells containing mostly rhodopsin (Figs. 5 and 6; Table II). This result is inconsistent with the hypothesis that the spontaneous isomerization of rhodopsin is the sole source of spontaneous quantum bumps and implicates metarhodopsin in the generation of these events. The simplest interpretation is that the reaction which inactivates metarhodopsin is reversible and that such reverse reactions can generate spontaneous quantum bumps. Thus, the results presented here lend

TABLE III

Computation of the Average Amplitude of Light-induced Quantum Bumps (A_L) from Measurements of the Average Amplitude of Spontaneous Bumps (A_s) and the Average Amplitude of Bumps During Dim Illumination‡ (A_i) under Conditions Where Most of the Pigment Was in the Metarhodopsin State*

	$\bar{A}_s \pm$ SEM	$\bar{A}_i \pm$ SEM	$\bar{A}_L$	$\bar{A}_s/\bar{A}_L$
	mV	*mV*	*mV*	
1	3.42±0.06	5.11±0.03	6.97	0.49
2	3.59±0.06	4.32±0.04	4.99	0.72
3	3.66±0.09	4.69±0.04	5.74	0.64
4	3.39±0.05	5.02±0.07	6.16	0.55
5	2.07±0.01	2.65±0.02	3.20	0.65
6	0.75±0.01	0.70±0.01	0.66	1.14

* $\bar{A}_L$ was computed from $\bar{A}_i$, $\bar{A}_s$, r_s (the rate of spontaneous bumps), and r_i (the rate of bumps during illumination) using the following formula: $f_s \bar{A}_s + f_L \bar{A}_L = \bar{A}_i$, where $f_L = (r_i - r_s)/r_i$ and $f_s = r_s/r_i$.

‡ To ensure that light-induced and spontaneous bumps were measured under the same conditions of adaptation, periods of light and dark were alternated at 10-s intervals.

experimental support to the theoretical arguments developed by Lamb (1981), which suggest that photoproducts may revert to the active state. Reverse reactions appear to be the primary source of spontaneous bumps in *Limulus* when a large fraction of the visual pigment is in the metarhodopsin state, but other sources of spontaneous quantum bumps are also likely to be important. Indeed, under conditions in which all of the visual pigment is in the rhodopsin state, the spontaneous quantum bumps cannot be due to reversion of metarhodopsin to the active state, and must be due either to the thermal isomerization of rhodopsin or to secondary reactions in the transduction cascade (see Fig. 1). The notion that spontaneous bumps can originate from more than one type of reaction and the possibility that the relative contribution of these reactions might differ from cell to cell may help to explain the wide quantitative variation in the fractional reduction in bump rate produced by the conversion of metarhodopsin to rhodopsin (Table II).

One difficulty with the idea that reverse reactions into the active state are responsible for quantum bumps is that spontaneous quantum bumps are smaller than light-induced ones (Table III). If there is a single active state of the pigment,

the response triggered by that state should be independent of how it is entered. One possible resolution to this problem is to assume that there is more than one active state, as diagrammed in Fig. 7*a*. In this model, absorption of light by rhodopsin leads to the formation of an active state, M_1^*, which then is converted to a second active (or partially active) state, M_2^*, through a relatively irreversible reaction. M_2^* is then converted into a completely inactive state by a subsequent

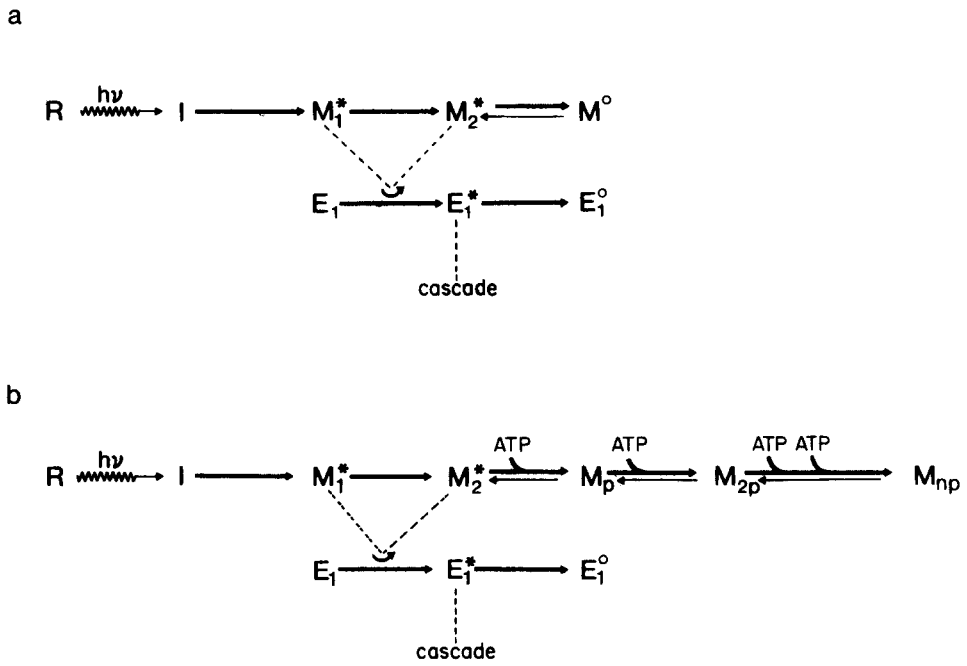

FIGURE 7. Models of quantum bump generation. (*a*) Similar to Fig. 1, except that there are two sequential active states. Reversion from M° to M_2^* generates a bump that is smaller than light-induced bumps, which are generated by passage through both the M_1^* and M_2^* states. M_1^* and M_2^* can both catalyze the conversion of E_1 and E_1^*. (*b*) Similar to *a*, except that the inactivation mechanism is more specific. A single phosphorylation reaction makes M_2^* inactive; subsequent phosphorylations reduce the probability of reversions of photoproduct to M_2^*.

reaction. Spontaneous reversal of the inactivation reaction would yield M_2^*, which in turn could be inactivated again without ever reverting to M_1^*. If the size of a quantum bump depended on the sum of the times spent in the active states, the spontaneous events that originate by reverse reactions from inactive metarhodopsin would be smaller than light-induced quantum bumps. Thus, reverse reactions make a "quasi-equivalent dark light" that evokes a response similar to, but smaller than, the response generated by forward reactions during real light. Biochemical evidence for multiple active states in rhodopsin is discussed below.

Although the data presented here suggest that inactivation of metarhodopsin is reversible, the switch that inactivates metarhodopsin is remarkably reliable when considered in quantitative terms. The steady state rate of spontaneous

quantum bumps (r) is ~1/s (Table II) under conditions where most of the pigment in the cell (~10^9 molecules) is metarhodopsin. Since the number of active metarhodopsin molecules is a negligible fraction of the total metarhodopsin, the number of inactive metarhodopsin molecules is ~10^9. Thus, the probability (per second) of a reverse reaction from inactive photoproduct to the active state must be $\leq 10^{-9}$, which implies that the inactivation switch is very reliable. The significance of the infrequent reverse reactions is a consequence of the fact that each reverting molecule generates a detectable signal.

Fig. 7 *b* shows how *Limulus* metarhodopsin might be inactivated using multiple phosphorylation reactions. This model is inspired by recent biochemical work (see below) suggesting that phosphorylation inactivates vertebrate metarhodopsin. It is assumed here that the final active state of metarhodopsin, M_2^*, is inactivated via a single ATP-utilizing phosphorylation reaction mediated by rhodopsin kinase, and that the reaction is at equilibrium (see below). There are then $n - 1$ further phosphorylation reactions. The equilibrium constant (K) for a single phosphorylation reaction is

$$K = \frac{[\mathrm{ATP}]}{[\mathrm{ADP}]} \frac{M_2^*}{M_p}, \tag{3}$$

where [ATP] and [ADP] are the concentrations of adenosine triphosphate and adenosine diphosphate, $\bar{M}_p$ is the average number of singly phosphorylated metarhodopsin molecules, and $\bar{M}_2^*$ is the average number of metarhodopsin molecules in the final active state. If there are $n - 1$ further phosphorylations having the same K, then the ratio of $\bar{M}^*$ to fully phosphorylated metarhodopsin ($\bar{M}_{np}$) can be shown to be

$$\frac{\bar{M}_2^*}{\bar{M}_{np}} = \left[K \frac{\mathrm{ADP}}{\mathrm{ATP}}\right]^n. \tag{4}$$

If we take the case where the rate of spontaneous quantum bumps (r) is due solely to the creation of M_2^* by dephosphorylation of inactive metarhodopsin via the reverse kinase reaction, then, from Eq. 4,

$$r = \frac{\bar{M}_2^*}{T} = \frac{\bar{M}_{np}}{T}\left[K \frac{\mathrm{ADP}}{\mathrm{ATP}}\right]^n, \tag{5}$$

where T is the average lifetime of M_2^*. It follows that the rate of spontaneous quantum bumps should vary directly with the inactive metarhodopsin concentration, as shown here, but vary inversely with the ATP concentration, as shown in the companion paper (Stern et al., 1985).

Eq. 5 can be solved for n if the equilibrium constant, K, for each phosphorylation reaction, the lifetime of M_2^*, and the ratio of ATP to ADP is known. The ATP/ADP ratio in most cell types is between 1 and 10. An upper limit on T is the duration of a quantum bump (100 ms). The K of rhodopsin phosphorylation is not known, but measurements on other proteins indicate that protein phosphorylation is surprisingly reversible (Rabinowitz and Lipmann, 1960; Shizuta et al., 1975; Fukami and Lipmann, 1983). For example, the phosphorylation of

casein by cAMP-dependent protein kinase has an equilibrium constant, K, of 0.042, which implies that only 1.9 kcal is lost during phosphorylation of this protein. Under conditions in which most of the pigment is in the inactive metarhodopsin state (i.e., $\bar{M}_{np} \cong 10^9$), the rate of spontaneous bumps (r) is ~1/s. Taking $T = 0.1$ s, ATP/ADP = 3, and $K = 0.042$, it follows from Eq. 5 that $n \simeq 5$; i.e., five phosphorylation reactions are required to inactivate metarhodopsin with the reliability that is experimentally measured.

In deriving the equations above, I have assumed that reactions are at equilibrium and that the only process by which metarhodopsin becomes dephosphorylated is by reverse reactions mediated by rhodopsin kinase (forming M_2^* and ATP). In addition, dephosphorylation may occur by a phosphatase reaction, yielding M_2^* and phosphate. Because this latter type of reaction may occur and because an upper limit of T has been used, $n = 5$ is a lower limit of the number of phosphorylation reactions required.

Within the context of the above model, the addition of one phosphate (or perhaps two if the $M_1^* \rightarrow M_2^*$ transition is via phosphorylation) is sufficient to inactivate metarhodopsin. The additional phosphorylations serve to decrease the probability that inactive metarhodopsin will spontaneously revert to the active state. In this regard, it is interesting that seven to nine phosphates are added to vertebrate metarhodopsin (Wilden and Kuhn, 1982), and preliminary work indicates that multiple phosphorylation also occurs in squid photoreceptors (Vandenberg and Montal, 1984). There is now evidence that rhodopsin phosphorylation is required to rapidly terminate the ability of metarhodopsin to activate phosphodiesterase in vertebrate rods (Sitaramayya and Liebman, 1983*a*, *b*). Recent work (Miller and Dratz, 1984) with this system indicates that more than one phosphorylation (but not all the phosphorylations) is needed to terminate the ability of rhodopsin to activate phosphodiesterase. This suggests that the first few phosphorylated states constitute a sequence of active states, and raises the possibility that the last few phosphorylations serve to prevent reverse reactions into the active state, as proposed here.

Relevance of Reverse Reactions in Vertebrate and Invertebrate Photoreceptors

From psychophysical experiments and measurements of pupil size, it appears that the vertebrate rod pathway is both excited and adapted by a slowly fading "dark light" during the recovery period following a bleaching light (Barlow, 1972; Alpern and Ohba, 1972). Direct recordings from rods demonstrate that bleaching lights produce a long-lasting afterpotential, and that during the final phase of the afterpotential there is a high frequency of spontaneous events resembling those induced by single photons (Lamb, 1980). In an elegant paper analyzing dark adaptation in rods, Lamb (1981) suggested that the "dark light" and its resulting desensitization are due to a high frequency of reverse reactions into the active state. Moreover, Lamb has shown that relatively simple assumptions regarding these reactions can lead to a coherent theory of dark adaptation. Consistent with this line of reasoning is the demonstration (Weinstein et al., 1967; Cornwall et al., 1983) that there is a permanent desensitization if photoproducts become stable (as occurs if the pigment epithelium is removed) and that

this desensitization can be abolished by reconverting photoproduct to rhodopsin by addition of 11-*cis* retinal (Pepperberg et al., 1978). However, the relationship between the spectroscopically identified photoproducts and the inactive pigment state capable of reverting back to the active state remains unclear (Brin and Ripps, 1977).

Similar ideas seem useful in explaining afterpotentials in invertebrate photoreceptors that follow moderate (Fein and Hanani, 1978) or bright lights (Figs. 4*b* and 5). In this regard, it is particularly interesting that the afterpotential that follows large-scale conversion of rhodopsin to metarhodopsin appears to be the summation of rapidly occurring quantum bumps (Minke et al., 1975) and that the afterpotential can be abolished by reconverting metarhodopsin to rhodopsin (Hochstein et al., 1973). A variety of explanations for these results have been proposed (see Hillman et al., 1983). However, the possibility that the afterpotential is due to reversions from inactive metarhodopsin has not been previously considered. After a bright light, multiple phosphorylation of the highly concentrated metarhodopsin could be a formidable strain on rhodopsin kinase and/or on the cell's ability to provide sufficient ATP. One might therefore expect considerable time to elapse before all metarhodopsins were fully phosphorylated. In the intervening period, the number of reverse reactions into the active state would be high. If, for example, there were a billion photoproduct molecules that were only doubly phosphorylated, it follows from Eq. 5 that there would be 1.5×10^6 spontaneous events per second, thus generating a bright "dark light."

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