

Nontransducing Rhodopsin

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ABSTRACT Rhodopsin is converted by light to an active photoproduct that triggers the transduction cascade. The active photoproduct must then be inactivated by some kind of chemical modification. The question addressed here is whether photoconversion of the inactive photoproduct to rhodopsin creates a modified form of rhodopsin that is unable to support transduction. This question was investigated in ultraviolet receptors of *Limulus* median eye by measuring the relative quantum efficiency of excitation after photoregeneration of rhodopsin from the inactive photoproduct. The results show that when this newly created rhodopsin absorbs a photon, no receptor potential is generated; i.e., the pigment is nontransducing. A dark process requiring 30–60 min returns rhodopsin to its transducing form.

INTRODUCTION

The absorption of light by rhodopsin leads to a series of conformational changes, resulting eventually in an active state that can trigger the transduction cascade. This active state of the visual pigment must then be turned off. Recent work on vertebrate rhodopsin has yielded substantial insight into the mechanism of this inactivation reaction. The active state, defined by the binding of G-protein or stimulation of phosphodiesterase activity, begins with the formation of the metarhodopsin II photoproduct (Bennett et al., 1982; Emeis et al., 1982). Inactivation occurs in several steps. Partial inactivation occurs as metarhodopsin becomes multiply phosphorylated (Liebman and Pugh, 1980; Sitaramayya and Liebman, 1983); further inactivation occurs after the binding of a 48-kD protein to phosphorylated metarhodopsin (Kuhn et al., 1984; Wilden et al., 1986). Finally, the conformational change from metarhodopsin II to metarhodopsin III or opsin completes the inactivation process (Bennett et al., 1982). Thus, three forms of chemical change appear to be involved in metarhodopsin inactivation.

Comparatively little is known about the inactivation of visual pigments in invertebrates, but the sequence homology of the vertebrate and invertebrate visual pigments (O'Tousa et al., 1985), the similarities of the light-dependent rhodopsin phosphorylation (Paulson and Hoppe, 1978), and G-protein activation (Saibil and Michel-Villaz, 1984; Vandenberg and Montal, 1984) make it likely that invertebrate and vertebrate phototransduction are closely related. One important difference between vertebrate rhodopsin and most invertebrate rho-

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dopsins is that the invertebrate photoproduct, metarhodopsin, is thermally stable: the *trans*-chromophore in metarhodopsin does not dissociate from the protein and rhodopsin is not regenerated in the dark. The only short-term mechanism by which rhodopsin can be regenerated from metarhodopsin is by a light-driven reaction that reisoimerizes the chromophore to the 11-*cis* configuration (Hubbard and St. George, 1958).

Because invertebrate metarhodopsin is thermally stable, the relative concentration of rhodopsin and metarhodopsin can be easily set and maintained, making invertebrate photoreceptors a useful preparation for the study of processes that depend on the pigment and its photoproducts. In a previous study (Lisman, 1985), the ultraviolet (UV) receptors of the *Limulus* median eye were used to study the inactivation reactions that occur after rhodopsin is converted to metarhodopsin. In this article, we have studied the reactions that occur after metarhodopsin is reconverted to rhodopsin by light. Our experiments are based on the following line of reasoning. Absorption of light by rhodopsin converts the pigment molecule to active metarhodopsin, which must then be inactivated by some type of pigment modification. The conversion of inactive photoproduct to rhodopsin by light should therefore yield a modified form of rhodopsin. If this modified rhodopsin then absorbs a photon, the pigment should pass directly to the modified, inactive state of the photoproduct without passing through the active state. Thus, one might expect there to be a nontransducing form of rhodopsin.

The hypothesis of nontransducing rhodopsin did not originate with us. It was first proposed by Hamdorf and Razmjoo (1977) and has since been incorporated into several models (Hamdorf, 1979; Paulson and Bentrop, 1984; Minke, 1984; Stieve, 1984). Hamdorf and Razmjoo showed that a model incorporating the assumption of nontransducing rhodopsin can explain the conditions under which afterpotentials occur in invertebrates (see Discussion). There have, however, been no previous attempts to demonstrate directly the existence of nontransducing rhodopsin. In this article, we describe conditions under which the absorption of light by rhodopsin fails to lead to a response, thus providing evidence for nontransducing rhodopsin. In addition, we describe the kinetics of the dark reaction by which nontransducing rhodopsin is converted to transducing rhodopsin. Preliminary reports of our findings have appeared (Lisman et al., 1985; Lisman and Goldring, 1985).

METHODS

The methods for recording and stimulating the UV receptors of the *Limulus* median eye were as described in Lisman (1985). Light was attenuated with neutral density interference filters. For exhaustive conversion of rhodopsin to metarhodopsin, a broad-band UV filter (UG11, Schott Glass Technologies, Inc., Duryea, PA) was used. For conversion of metarhodopsin to rhodopsin, an orange cut-on filter (OG530, Schott) was used in combination with a yellow cut-on filter (GG495, Schott). The purpose of this second filter was to provide added blocking of short-wavelength light that might be absorbed by rhodopsin. To measure the relative quantum efficiency, the preparation was exposed to dim light that was restricted to the UV using a narrow-band 380-nm interference filter.

The data for determination of the relative quantum efficiency of excitation were

collected by alternating ~8-s dim UV light pulses with ~8-s periods of darkness. Data were recorded on chart paper at a speed of 25 mm/s for subsequent analysis. To compute the quantum bump rate during light, the total number of bumps during a group of light pulses (usually 12) was summed and then divided by the total period of illumination (minus a small correction for the latent period). To compute the rate of spontaneous bumps (Adolph, 1964), the number of bumps in the dark was measured and divided by the period in the dark (the first second after the termination of each light pulse was omitted to ensure that long-latency light-induced bumps would not be counted as spontaneous bumps). The total period used to determine the rate of spontaneous bumps was four times longer than the period used for determining the rate of light-induced bumps. This strategy was chosen in order to increase the total number of bumps used to determine the dark rate, thereby reducing the error in determination of the dark rate. This strategy was justified because the drifts in the spontaneous quantum bump rate were small and slow. To compute the relative quantum efficiency, the spontaneous rate was subtracted from the rate during light and this difference was divided by the relative light intensity. Successful experiments required stable recordings for 5–6 h. Furthermore, cells that generated only small quantum bumps (2 mV maximum) were discarded because of the difficulty of accurately counting the bumps. The overall success rate in doing experiments that yielded useful data was ~10%.

The artificial seawater used in all experiments contained 10^{-6} M tetrodotoxin in order to abolish the small action potentials that can be recorded from UV photoreceptors (Nolte and Brown, 1972a). It was useful to abolish these action potentials so as not to confuse them with small quantum bumps. Experiments were conducted at ~18°C.

RESULTS

The changes of experimental interest were those that occurred after inactive metarhodopsin was converted to rhodopsin by light. The protocol for studying these changes is illustrated in Fig. 1. Rhodopsin ($\lambda_{\max} = 360$ nm) was converted to metarhodopsin ($\lambda_{\max} = 470$ nm) using exhaustive UV irradiation. This stimulus evoked a saturating receptor potential and a prolonged depolarizing afterpotential (PDA), as previously described (Nolte and Brown, 1972a; Hochstein et al., 1973). The PDA slowly declined toward the baseline. After ~1 h, the PDA consisted of many superposed quantum bumps. Over the next several hours, the rate of these bumps slowly declined (Lisman, 1985). During this decline, there was little or no regeneration of metarhodopsin to rhodopsin. Under these conditions, most of the pigment was in the inactive metarhodopsin state, but there was still some residual rhodopsin. When the cell was exposed to dim UV pulses, absorption of photons by this residual rhodopsin generated quantum bumps. These responses were used to measure the light-induced quantum bump rate (see Methods). Cells were then exposed to a bright orange light (the regenerating light) to photoregenerate rhodopsin. The cell was then exposed to dim UV pulses in order to probe the excitability of the newly created rhodopsin.

If the rhodopsin newly formed by the regenerating light can support transduction, a stimulus should evoke more quantum bumps after the regenerating light than before it. We found, however, that the rate of quantum bumps induced by UV pulses of fixed intensity was not immediately affected by the regenerating light. This is illustrated in Fig. 2. Trace *a* shows the responses of the cell to dim UV light under conditions where the pigment was mostly in the metarhodopsin

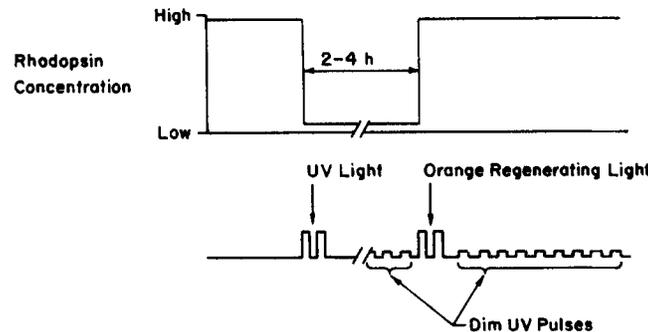


FIGURE 1. Experimental protocol. Bright UV stimulus converts most of the rhodopsin to metarhodopsin. After 2–4 h, the cell dark-adapts to the point where quantum bumps can be measured in response to dim UV pulses. An orange light is then used to photoregenerate rhodopsin. Subsequent quantum efficiency changes are monitored using dim UV pulses.

state. From many such traces, it was determined that the light-induced quantum bump rate in this cell was 1.6/s. Trace *b* shows the responses to a pair of regenerating stimuli. Trace *c* illustrates the response to the same UV pulses given shortly (a few minutes) after the regenerating light. Although the spontaneous rate was clearly lower than before the regeneration of rhodopsin, as previously reported (Lisman, 1985), the light-induced rate was 1.6/s, identical to that before the regeneration of rhodopsin. This close match was certainly coincidental, since the accuracy of our measurements was no better than 15%. However, the change in rhodopsin concentration was very large (see Table I). Thus, if the newly

TABLE I

	1A	1B	2	3	4	5	6
ΔQE	14	10	27	43	11	5.3	3.3
\bar{V} (mV)	3.2	4.1	3.1	2.6	3.0	2.6	—
T_m (min)	198	192	180	78	300	264	138
T_R (min)	139	118	103	66	89	50	47
$T_{1/2}$ (min)	22.5	42	63	37	37	—	—
r_s (s^{-1})	0.2	0.3	0.57	0.18	0.18	0.16	0.7
Sigmoid	?	?	Yes	Yes	Yes	Yes	?

ΔQE is the ratio of relative quantum efficiency before and after recovery. \bar{V} is the average quantum bump amplitude. T_m is the total time in the high-metarhodopsin state before regenerating light. T_R is the total time in the high-rhodopsin state after orange regenerating light. Short times for cells 5 and 6 were due to loss of impalement. Since complete recovery of QE may not have occurred in these cells, the ΔQE given is a lower limit. $T_{1/2}$ is the time to half-maximal recovery of QE. r_s is the rate of spontaneous bumps in the high-rhodopsin state. "Sigmoid" describes the shape of the QE recovery curve. The ΔQE values for cells 1–4 provide an estimate for the change in the rhodopsin concentration produced by exhaustive UV bleaching. Residual rhodopsin would be expected at photoequilibrium because of absorption of UV light by the β -band of metarhodopsin.

created rhodopsin could have supported transduction, large and easily detectable changes in the number of bumps induced by the dim UV pulses should have occurred. We can thus conclude that the newly created rhodopsin cannot generate a quantum bump in response to photon absorption; i.e., the rhodopsin is nontransducing.

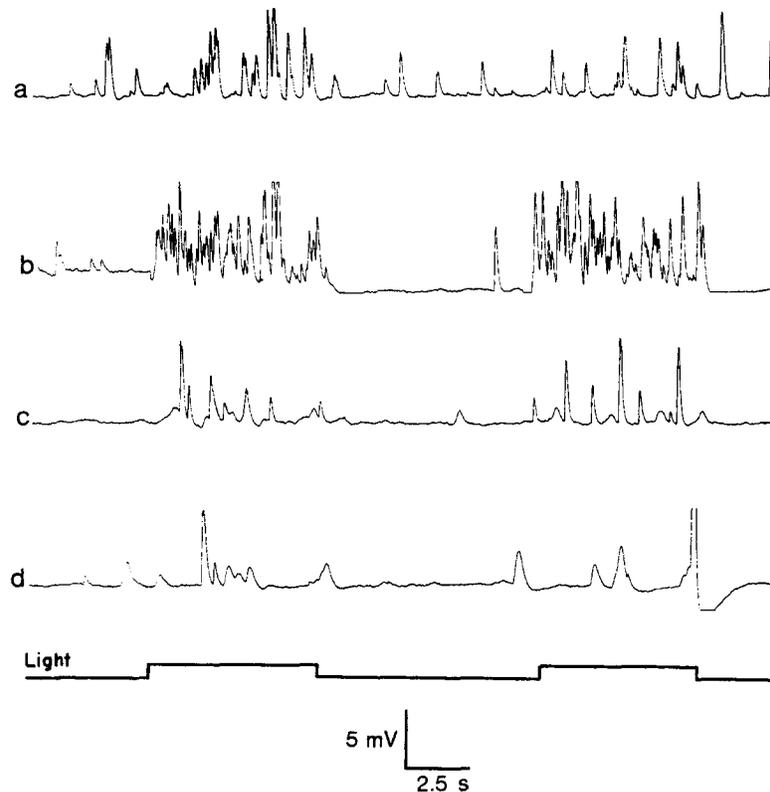


FIGURE 2. Spontaneous and light-induced responses illustrating the type of data from which quantum efficiency calculations were made. (a) Responses to two sequential dim UV light pulses under conditions where most of the pigment is in the metarhodopsin state (3 h after conversion of rhodopsin to metarhodopsin). Note the high rate of spontaneous bumps between light pulses. (b) Responses to two bright orange regenerating lights. (c) Responses to same dim UV flashes as in *a* given 3 min after the regenerating light put ~100% of the pigment into the rhodopsin state. The rate of light-induced quantum bumps is close to that in *a*. (d) Responses to dimmer UV light pulses 26 min after the regenerating light. The intensity of light was ~30 times lower than in *a* and *c*, but the number of bumps produced was approximately the same.

The quantum bumps evoked by dim UV light and the spontaneous quantum bump rate were then monitored over the next several hours. The spontaneous quantum bump rate changed only slightly with time (Fig. 3); the slow, small drifts that occurred were not consistent from cell to cell. During this period,

however, there was a dramatic increase in the number of bumps evoked by the dim UV light. For the light-induced quantum bump rate to be computed accurately, superposition of bumps must occur infrequently. To satisfy this condition, it was necessary to reduce the intensity of the dim UV light as the quantum efficiency increased. An example of the responses used to compute the relative quantum efficiency many minutes after the reconverting light is shown in Fig. 2, trace *d*. The number of bumps evoked by light was roughly the same as just after the regenerating light (trace *c*); however, the light intensity had been reduced by a factor of 27. Thus, the relative quantum efficiency had risen enormously. Fig. 4 shows how the relative quantum efficiency increased with time after the regenerating light. The details of computation of the relative quantum efficiency are given in the Methods. The results from two cells are shown to give an indication of the variability of the results. The relative quantum

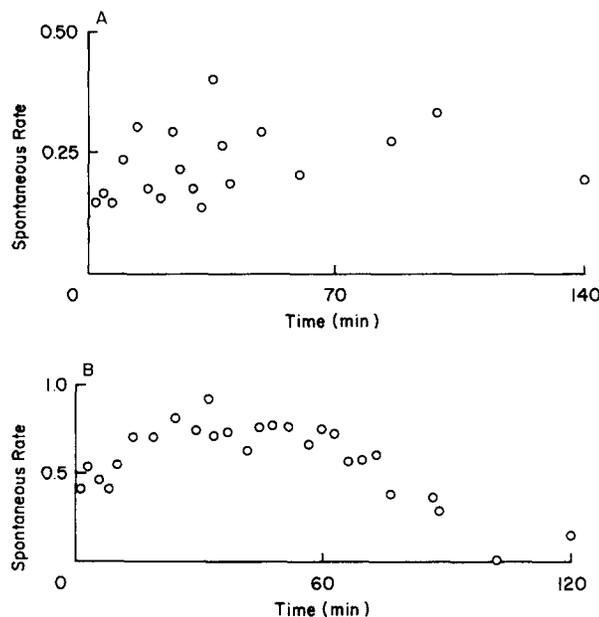


FIGURE 3. Rate (s^{-1}) of spontaneous bumps as a function of time after regenerating light. The data in *A* and *B* are from two different cells (experiments 2 and 1A, respectively, of Table I).

efficiency slowly increased, eventually reaching a value about an order of magnitude higher than the initial value. The actual increases in Fig. 4, *A* and *B*, are 27 and 14, respectively. The recording illustrated in Fig. 4*B* was particularly stable and it was possible to repeat the whole protocol again on this same cell, with similar results (Table I). Data from other cells are also given in Table I. The half-time of the recovery of quantum efficiency was typically ~ 30 min (Fig. 4*A*), but could be as long as 60 min (Fig. 4*B*). A striking aspect of the recovery process in four of seven cells was that the recovery kinetics were highly sigmoidal (*A*). In other cells (three of seven), the recovery was not clearly sigmoidal (*B*), but sigmoidal kinetics could not be excluded.

The computation of relative quantum efficiency relies on measurements of the number of quantum bumps during a particular period and does not involve

consideration of the size of bumps. However, if the size of the bumps decreased, as occurs during light adaptation, the signal-to-noise ratio would fall and detection of bumps would be impaired. It was therefore important to consider whether the reduction in quantum efficiency that we observed after a regenerating light might be due to an error in counting bumps caused by the adapting effect of the regenerating light. Four observations indicate that such an error was not significant. First, the waveshape (Fig. 2, trace *b*) of the response to the regenerating light did not have an initial transient phase, which indicates that the reconverting

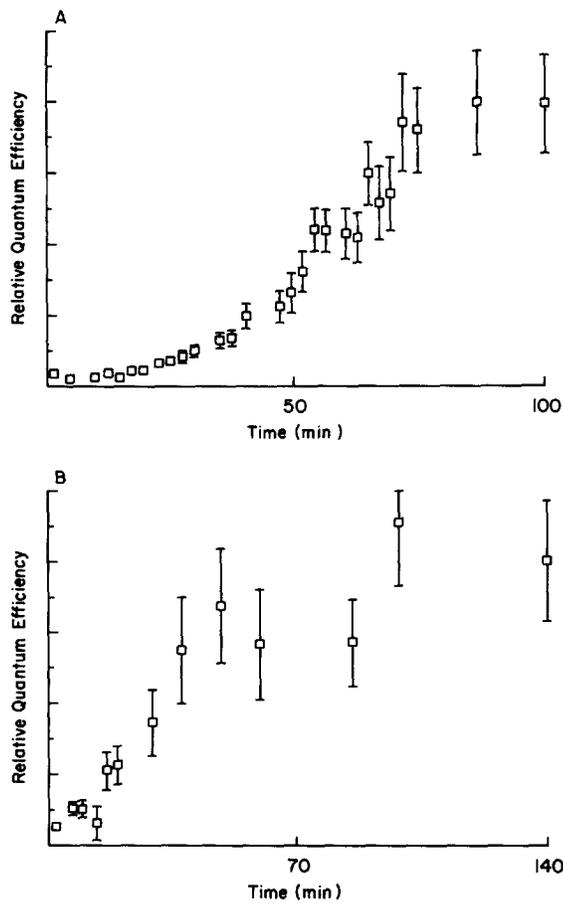


FIGURE 4. Quantum efficiency of excitation as a function of time after the regenerating light. Error bars indicate the standard deviation. Where error bars are not shown, the error was less than the width of the symbol. Data are from the same cells as in Fig. 3.

light did not light-adapt the cell (Lisman and Brown, 1975). Second, the average amplitude of quantum bumps was not affected by the regenerating light, which again indicates that light adaptation did not occur (data not shown). Third, the rate of spontaneous bumps did not rise significantly during the recovery period (Fig. 3). If the cell had become light-adapted in such a way as to lead to the uncountability of some bumps, the countability of spontaneous bumps should also have increased as the cell dark-adapted. Finally, a more general type of control experiment was done in which the cell contained primarily rhodopsin

even before the regenerating light was given. The relative quantum efficiency was measured as a function of time after the regenerating light and was found not to change. Thus, we conclude that the changes in quantum efficiency of the kind shown in Fig. 4 occurred because of time-dependent changes in the production of bumps and not in their detectability. It should be noted that *Limulus* UV receptors are uniquely suited to the type of experiment reported here because the λ_{\max} values of rhodopsin and metarhodopsin are separated by >100 nm. For this reason, a bright orange light that reconverts metarhodopsin to rhodopsin stimulates so few of the residual rhodopsin molecules that the cell remains completely dark-adapted.

It was of interest to examine the early receptor potential (ERP) during the period after a regenerating light to see if there were any changes in the ERP that paralleled the changes in quantum efficiency. Previous work showed that a flash that converts rhodopsin to metarhodopsin generates a negative ERP, whereas a flash that converts metarhodopsin to rhodopsin generates a positive ERP (Lisman, 1985). Fig. 5 shows that the regenerating light creates a pigment

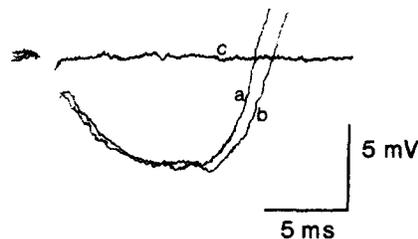


FIGURE 5. ERPs evoked by white light when the cell was in the all rhodopsin state. ERP 1 was recorded 5 s after a regenerating light converted inactive metarhodopsin to rhodopsin. ERP 2 was recorded 20 min after a similar regenerating light. During this period, in which there were no changes in the ERP, there were large changes in the quantum efficiency of excitation.

state, which, when exposed to bright flashes, generates ERPs with the negative polarity characteristic of rhodopsin. The two traces in Fig. 5 are from separate runs of this experiment on the same cell; the ERP was evoked either 5 s or 20 min after the regenerating light. The two ERPs are essentially identical (note that the rapidly rising component at the right of the waveform is due to the late receptor potential). The conclusion to be drawn from the similarity of these ERPs is that the reconvert light regenerates rhodopsin rapidly, a conclusion consistent with what is generally observed for invertebrate visual pigments (reviewed by Hillman et al., 1983). Moreover, during the many minutes following the regenerating light, there are no changes in the amount of rhodopsin, in the quantum efficiency of isomerization, or in the nature of the pigment transitions as reflected by the ERP waveform. Thus, the modifications of the pigment that affect its ability to transduce do not appear to affect the generation of the ERP.

DISCUSSION

Our principal conclusion is that photoregeneration of rhodopsin from inactive metarhodopsin produces a form of rhodopsin that is incapable of initiating

transduction. This nontransducing rhodopsin is then converted to a transducing form by a dark process that requires 30–60 min to reach completion. A striking aspect of this recovery in some cells was the sigmoidal kinetics of the process (Fig. 4A and Table I). If the recovery of transducing rhodopsin required only a single chemical process governed by an invariant rate constant, the recovery kinetics would initially be linear and then would saturate along an exponential curve. The observed sigmoidal kinetics exclude this class of models and suggest that the conversion of nontransducing rhodopsin to transducing rhodopsin is a multistep process.

Fig. 6 summarizes our current understanding of the light-dependent modifications of *Limulus* rhodopsin and metarhodopsin. The scheme has some elements in common with those proposed by Hamdorf and Razmjoo (1977), Paulson and

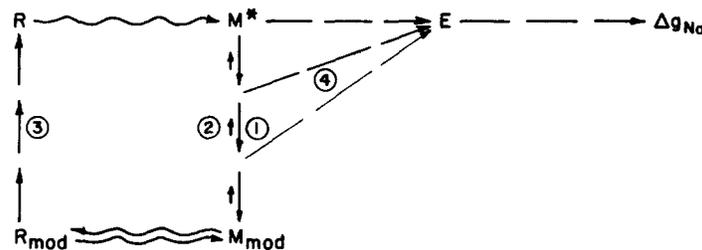


FIGURE 6. Scheme illustrating the multistep character of the pigment inactivation and reactivation reactions. Rhodopsin is converted by light to an active photoproduct, M^* , that can stimulate the enzyme cascade, E , leading to a change in Na conductance, Δg_{Na} . M^* is inactivated by multiple reactions (1) leading to a modified metarhodopsin, M_{mod} . Spontaneous discrete events owing to inactive photoproduct may be caused by spontaneous reversion of M_{mod} back to M^* (2) or by partially inactivated metarhodopsin that can stimulate E with low probability (4). If M_{mod} is converted to rhodopsin by light, the rhodopsin is in a modified form, R_{mod} , which is then demodified by multiple dark reactions (3). This scheme has some elements in common with the scheme proposed by Hamdorf and Razmjoo (1977) and later modified by Paulson and Bontrop (1984) and Stieve (1984).

Bontrop (1984), and Stieve (1984). The scheme is presented here to emphasize that both the inactivation of metarhodopsin and the reactivation of rhodopsin may be multistep processes. The evidence that rhodopsin reactivation occurs in multiple steps was described above. The argument (Lisman, 1985) that inactivation of metarhodopsin occurs in multiple steps is based on two properties of the PDAs that follow bright lights in invertebrate photoreceptors. First, the PDA can be abolished by eliminating metarhodopsin (Nolte and Brown, 1972b; Hochstein et al., 1973); thus, the afterpotential is due to events at the pigment level rather than to a latching of a step downstream from the pigment. Second, the PDA is the superposition of discrete waves similar to those produced during light (Minke et al., 1975); the decline of the PDA is due to a reduction of the rate of these waves (Lisman, 1985). These results can be explained (Lisman,

1985) on the assumption that metarhodopsin inactivation is a graded, multistep process; as metarhodopsin undergoes further steps in the inactivation process, the probability that a given metarhodopsin will produce a quantum bump goes down, and the PDA thus returns toward the baseline.

Mechanism of Inactivation and Its Reversal

Our measurements imply that there are different chemical forms of rhodopsin but place no restrictions on how these forms differ. For instance, it is possible that the functional changes in the pigment are mediated entirely by conformational changes, the free energy of which comes from dissipation of stored photon energy (Cooper, 1981). The absorption of light by inactive metarhodopsin might yield a conformation of rhodopsin that cannot support transduction, but which slowly decayed into a conformation that can. The main argument against this idea is that conformational changes in visual pigments are usually associated with changes in the ERP (see Cone and Pak, 1971), but no changes in the ERP occur in the transition from nontransducing to transducing rhodopsin (Fig. 5).

Alternatively, the changes in the transducing state of rhodopsin may be due to phosphorylation or dephosphorylation of the pigment (Paulson and Bontrop, 1984; Lisman, 1985). There is no evidence yet that the observed light-dependent phosphorylation of invertebrate metarhodopsin inactivates the pigment, but the work on vertebrate rhodopsin makes this a plausible idea (see Introduction). In terms of this model, photoregeneration of invertebrate rhodopsin from phosphorylated metarhodopsin would yield phosphorylated rhodopsin, which, upon absorbing another photon, would be transformed to inactive metarhodopsin without passing through the active state; i.e., the pigment would be nontransducing. Restoration of transducing rhodopsin would require dephosphorylation of rhodopsin. Such an explanation of our results is qualitatively consistent with the recent work on the phosphorylation of fly visual pigment from Paulson and Bontrop (1984). This work shows that creation of metarhodopsin makes the pigment a target for phosphorylation; metarhodopsin stays phosphorylated until it is photoregenerated to rhodopsin, whereupon the rhodopsin is dephosphorylated by a dark process. To evaluate the possibility that this dephosphorylation of rhodopsin underlies the conversion of nontransducing rhodopsin to transducing rhodopsin, it would be desirable to compare the time courses of the two processes in the same species, but such data are not yet available. The dephosphorylation reaction *in vitro* in fly occurs in ~1 min, much faster than the 30–60 min required to restore the transducing ability of *Limulus* UV pigment (Fig. 4).

Physiological Significance of Nontransducing Rhodopsin

As first suggested by Hamdorf and Razmjoo (1977), the existence of nontransducing rhodopsin can explain an interesting property of PDAs in invertebrate photoreceptors. Conversion of a large fraction of rhodopsin to metarhodopsin is followed by a PDA that slowly decays to baseline. If the rhodopsin is then photoregenerated, it is not immediately possible to induce another PDA by stimulating this newly made rhodopsin (Hochstein et al., 1973). If, however, stimulation is delayed many minutes, it becomes possible to induce a PDA.

Hamdorf and Razmjoo (1977) proposed that PDA induction requires that a large, critical fraction of the total pigment be in the active state (reviewed in Hillman et al., 1983). If newly regenerated rhodopsin cannot be put into the active state because it is nontransducing, PDAs cannot be induced.

The role of nontransducing rhodopsin discussed above concerns responses to bright, colored lights; it is of interest to consider whether nontransducing rhodopsin might be important during more general types of illumination. If a cell were exposed to bright, broad-band illumination, rhodopsin and metarhodopsin would be rapidly interconverted and their concentrations would be roughly equal. The pigment would become modified in the metarhodopsin state and then demodified after photoregeneration. If modified (nontransducing) rhodopsin absorbed a photon, no transduction would occur, and the quantum efficiency would be reduced, contributing to a form of light adaptation. How much nontransducing rhodopsin actually accumulates during bright broad-band lights remains to be determined.

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