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Early Receptor Potential: Photoreversible Charge Displacement in Rhodopsin

Abstract. *When the eye is illuminated by an intense flash, the visual pigment rhodopsin begins to pass rapidly through a series of intermediate states, eventually becoming bleached. If a second flash is delivered during the lifetimes of these intermediates the rhodopsin can be photoregenerated. A fast electrical response of the visual receptors, the early receptor potential, is elicited by the first flash. A similar response is elicited by the second flash, but the polarity of this response is reversed. Moreover, this response can be separated into three components, each arising from the action of light on a different intermediate. It is likely that all these fast responses, including the early receptor potential, arise from charge displacements in the visual-pigment molecule.*

The absorption of a single photon by the visual pigment rhodopsin can excite a rod (*1*). We do not yet know how the excitatory signal is initiated nor how it is transmitted to the synaptic end of the receptor. Recently, by using intense light flashes, Brown and Murakami discovered in the retina a new electrical response with no detectable latency (*2*). They named this response the early receptor potential (early RP) to distinguish it from a later electrical response, which is presumably generated at the synaptic end of the receptor. The later response arises just before the early RP is fully completed.

The early RP is a biphasic response consisting of an initial cornea-positive phase (R1) followed by a slower cornea-negative phase (R2), lasting for a few milliseconds after the flash (*3, 4*). These two phases can be isolated by cooling the eye to near-freezing temperatures, in which case R1 remains but R2 is reversibly abolished (*5*). The spectral sensitivities of both R1 and R2 match the absorption spectrum of rhodopsin, and the amplitude of each phase is linearly proportional to the fraction of rhodopsin bleached by the stimulus flash (*3, 5*). These characteristics strongly imply that the early RP is generated by processes closely linked to rhodopsin.

That the early RP is not generated by changes in membrane permeability which in turn initiate passive ionic currents has now been almost conclusively demonstrated (*5–7*). Therefore, the early RP is most likely generated by the net displacement of electric charge (electrons, ions, and charged groups) as rhodopsin or neighboring molecules undergo changes in configuration. Since only R1 survives freezing temperatures, the early RP probably arises from two independent or sequential charge displacements (*5*). Brindley and Gardner-Medwin have suggested that changes in the waveform of the early RP produced by salts and by glycerol can all be accounted for by probable changes in the electrical filtering characteristics of the retina, if it is assumed that the early RP is generated by two successive movements of charge (*7*). That the first charge displacement must originate in or very near the rhodopsin molecule is indicated by the rapidity with which R1 arises during the stimulus flash. Using a spark gap with a flash duration of 0.7 μsec, I have found no indication of a latent period between the flash and the rising phase of R1 (*8*). Any latent period that does exist is definitely shorter than 0.5 μsec at 25°C. In addition, as will become clear from what follows, both R1 and R2 develop concurrently with the initial stages of the bleaching process in rhodopsin. Therefore, the presumed charge displacements that generate these responses must be intimately related to the bleaching process in the rhodopsin molecule.

A photon initiates the bleaching process in rhodopsin by stereoisomerizing the 11-*cis* chromophore of rhodopsin to the all-*trans* configuration (pre-lumirhodopsin) (*9*). The molecule then proceeds through a series of intermediate states (lumi, meta I, and meta II) to the end products, retinal and opsin. Each intermediate is a strongly absorbing pigment. This is of primary concern because, by exposing these intermediates to light, the course of bleaching can be manipulated. In particular, rhodopsin can be photoregenerated. Light can apparently isomerize the chromophore of any of these intermediates to the *cis* configuration present in rhodopsin or isorhodopsin (*10, 11*), and this isomerization initiates a reaction leading back to rhodopsin or isorhodopsin (*9*). Clearly, if the early RP depends closely upon rhodopsin, it too should be photoregenerated.

The photoregeneration of the early RP is shown in Fig. 1. The responses shown in this figure were obtained from excised intact eyes of the albino rat. Saline solutions were used to make electrical contact with the cornea and back of the eye. Light was delivered to the eye through a microscope condensing lens to provide uniform illumination of the entire retina, and stimulus flashes lasting 150 μsec were produced by a xenon flash tube. The width of the amplifier band was set at 0.1 to 10,000 hz. All electrical and photovoltaic artifacts were eliminated by appropriate shielding; and, since albino eyes were used, all observed responses can be attributed to the visual pigment in the retina (*3, 12*). For the test flash in Fig. 1, an interference filter with peak transmission at 560 nm was placed in the stimulus beam. With this wavelength the test flash elicited responses primarily from rhodopsin, because at the times when this test flash was delivered only long-lived intermediates would be present, and these absorb maximally in the blue region of the spectrum. Each test flash bleached about 10 percent of the rhodopsin present in the eye.

The top trace in Fig. 1 was obtained from the first test flash presented to the dark-adapted eye. It shows the typical biphasic waveform of the early RP, an initial positive phase (R1) followed by a slower negative phase (R2). After this response was obtained, the eye was exposed to strong tungsten light through a cutoff filter that eliminated all wavelengths shorter than 520 nm. This source delivered at least one photon to each rhodopsin molecule within 10 seconds. Hence, after a 1-minute exposure to this light, little or no rhodopsin remained in the eye. I veri-

fied this by presenting the second test flash, which did not elicit an observable early RP even though the amplifier gain was increased to show the noise level of the preparation. Approximately 15 seconds after the tungsten light was turned off, an intense blue flash was delivered to the retina to photoregenerate rhodopsin from the intermediates still present. A few seconds after this blue flash, the third test flash was presented. The third test flash elicited a large early RP with an essentially normal waveform (Fig. 1). To be sure that this regenerated response was not simply the result of waiting in the dark, I obtained a control trace from a second eye subjected to the same three test flashes and 1-minute bleaching exposure, but the blue flash was omitted. It is clear from the absence of a significant response in the control trace that the early RP in the first eye must have been photoregenerated by the blue flash. This photoregeneration of the early RP can be repeated many times if each exposure to the tungsten light is followed by an intense blue flash. In an experiment somewhat similar to that shown in Fig. 1, Arden, Ikeda, and Siegel (*13*) demonstrated that, after the eye is exposed to tungsten light, the amplitude of the early RP increases when a series of white stimulus flashes are presented. Moreover, they verified, by means of densitometry, that these white flashes also photoregenerate pigment.

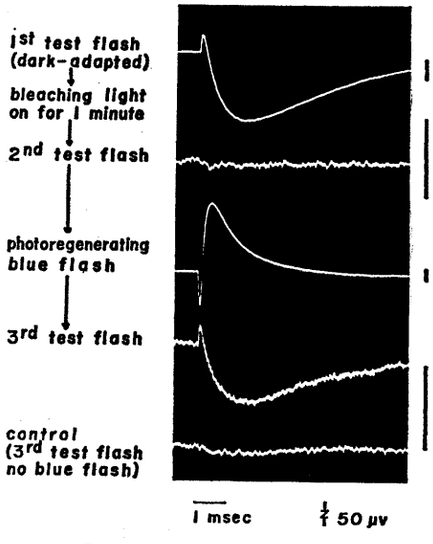

Fig. 1. Photoregeneration of the early RP in the eye of the albino rat. Both the test flash and the bleaching light consisted of long wavelengths primarily absorbed by rhodopsin. The blue, photoregenerating flash contained wavelengths absorbed by the longer-lived intermediates of the bleaching process. The control trace was obtained from a second eye subjected to the same bleaching exposure and test flashes, but without the blue flash. Temperature, 27°C.

The photoregenerating blue flash elicits a large, biphasic response similar to the early RP, but of opposite polarity (Fig. 1). Arden, Ikeda, and Siegel reported finding a fast negative response similar to the first phase of the blue-flash response in Fig. 1 and demonstrated that this fast negative response is associated with the photoregeneration of rhodopsin (*13*). They suggest that it is produced by both meta I and meta II when these intermediates absorb light and revert to rhodopsin. It is clear from the blue-flash response in Fig. 1 that a slower positive response may also be associated with photoregeneration of rhodopsin. In order to interpret these new responses one must identify the photoproducts on which they depend.

The kinetics of bleaching in rhodopsin differ somewhat when observed in solution instead of in the eye (*14, 15*), and the characteristics of each intermediate differ somewhat among animals (*9*). Hence, the best way to identify the photoproducts on which these new responses depend is to observe both the responses and the bleaching intermediates under similar conditions. Fortunately, Hagins has studied the bleaching process of rhodopsin in the intact eye of the albino rabbit (*15*). He investigated the process with reflection densitometry of the retina following flash-illumination and found that, in the temperature range above freezing, three different bleaching intermediates could be clearly differentiated. He labeled these intermediates A, B, and C in the order of their appearance following a saturating flash of light (a flash that delivers at least one photon to every rhodopsin molecule). Intermediate A, the first intermediate discernable with his apparatus, absorbs maximally at somewhat shorter wavelengths than rhodopsin. This intermediate forms in less than a few milliseconds at 5°C and decays with a half-time of about 60 msec to the second intermediate, intermediate B. This decay is a first-order process, so the half-time of formation of intermediate B is also about 60 msec at 5°C. Intermediate B probably consists of a mixture of pigments because only part of it decays to the third intermediate, intermediate C. The half-time of this decay is several minutes at room temperature. Intermediate C absorbs maximally at about 485 nm. It is therefore easily confused with metarhodopsin I, especially because, as Hagins found, intermediate C is formed immediately if intermediate B is exposed to light. However, in the dark, the formation time of intermediate C is several orders of magnitude slower than that of meta I. When Hagins' observations on the intact rabbit eye are compared with later work on cattle rhodopsin in solution (*11*), it appears that intermediates A, B, and C probably correspond primarily to meta I, meta II, and a later intermediate that absorbs maximally at 465 nm.

To work out the relationship between the photoproduct responses and the bleaching intermediates, I have obtained photoproduct responses from the albino rat eye under conditions that closely simulate those used by Hagins. As a result, I have found that for each of his three intermediates there is a corresponding response. There are shown in Fig. 2 and are labeled A, B, and

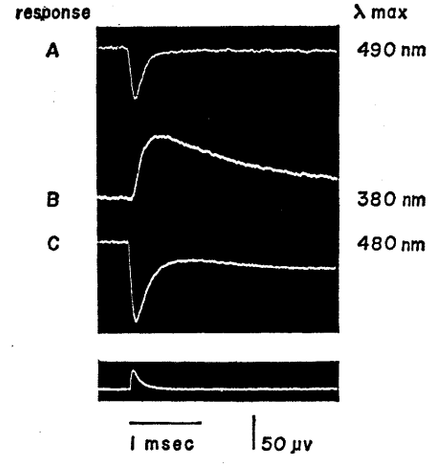

Fig. 2. Photoproduct responses obtained at three different times in the bleaching process in the eye of the albino rat. Response A was obtained at 5°C with a white flash delivered 50 msec after a saturating flash had been presented to the dark-adapted eye. Responses B and C were obtained at 27°C following a 30-second bleaching exposure to tungsten light. Response B was obtained with a narrow-band blue flash with a maximum at 400 nm immediately following the bleaching exposure; response C was obtained with a white flash delivered 3 minutes after the bleaching exposure. Response C is slightly distorted by the presence of a small early RP because a small amount of rhodopsin regenerated after the bleaching exposure. Each response was obtained from a different eye. The wavelength of maximum sensitivity for each response is shown on the right. Flash duration is shown by a photodiode trace. Recording conditions are the same as in Fig. 1.

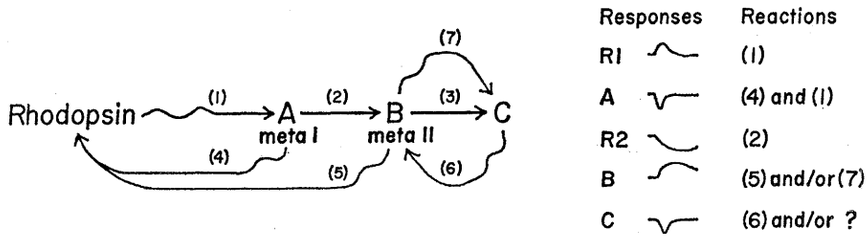

Fig. 3. Summary of the photochemical and thermal reactions of rhodopsin in the intact eye, as discussed in the text. Photochemical reactions are denoted by wavy lines; thermal (dark) reactions, by straight lines. The five electrical responses so far identified are listed along with sketches to show their polarity and waveform and the probable reactions on which each depends.

C to emphasize this relationship. When a dark-adapted eye is cooled to 5°C and then exposed to a saturating flash, the early RP elicited by this flash consists of a single positive phase, R1 (at this temperature R2 is absent). If a second flash is presented to the eye within a fraction of a second, it elicits a fast negative response, response A. One can determine the formation and decay times of this response by changing the time interval between the two flashes and presenting each pair of flashes to fresh, dark-adapted eyes. At 5°C, the half-time of formation for response A is less than 30 μsec, and the half-time of decay is about 0.5 second. As the interval between the two flashes is increased, the amplitude of response A gets progressively smaller, and a slower positive response develops, forming with the same half-time as the half-time of decay of response A. This positive response, response B, decays very slowly at 5°C, though more rapidly at higher temperatures. At room temperature, the half-time of formation of response B is about 1 msec, and it decays within a few minutes. As intermediate B decays, a fast negative response develops, response C. Response C is formed more rapidly if the dark-adapted eye is exposed to intense tungsten light instead of to the initial saturating flash. In this case response C is well developed immediately after the exposure to light, and it grows within a few minutes to become the dominant response (Fig. 2).

To establish further the relationship between these electrical responses and the intermediates of bleaching, I obtained the action spectrum of each response. To avoid distortions by spurious photoproducts, I used a fresh eye for each observation; to obtain responses of sufficient amplitude, I used broad-band interference filters (one-tenth transmission at ± 4 percent λ_{max}). The wavelength of maximum sensitivity for each response is shown in Fig. 2 and is estimated to be accurate to within ± 10 nm. Response A exhibits essentially the same formation and decay times, temperature dependence, and spectral sensitivity as Hagins found for intermediate A. This same correspondence holds for response B and intermediate B and for response C and intermediate C. It seems apparent, therefore, that responses A, B, and C arise from the action of light on these three intermediates (*16*).

If a net displacement of charge occurs when rhodopsin is converted to any of these intermediates, then a similar net displacement of charge should occur when this intermediate is photoconverted back to rhodopsin. This should be true even if the reaction paths and rates differ between the forward and back reactions. At 5°C, as rhodopsin absorbs light and goes to intermediate A, a fast positve response (R1) is observed; when intermediate A absorbs light and goes back to rhodopsin a fast negative response (response A) is observed. Similarly, at higher temperatures, during the time in which intermediate A decays to intermediate B, a slow negative response arises (R2), whereas when a fraction of intermediate B on absorbing light goes back to form rhodopsin, a slow positive response is observed (response B). Hence, responses R1 and A probably arise from the same reversible reaction going in opposite directions, and similarly, responses R2 and B may arise from a second reversible reaction.

No response has yet been identified that corresponds to the thermal decay of intermediate B to intermediate C. This may be because the decay is very slow, and slow electrical responses are likely to be filtered out in the retina. However, the occurrence of response C suggests that the photoconversion of intermediate C is a rapid, charge-displacement reaction. This further suggests that response B may arise from the rapid photoconversion of intermediate B either to rhodopsin or to intermediate C, or possibly to both.

Figure 3 summarizes the photochemical and thermal reactions discussed here and indicates the reactions that probably generate responses R1, R2, A, B, and C. Little is known about the specific charge displacements that might occur in these reactions. But there is one piece of evidence that may well bear upon the presumed charge-displacement reaction for responses R2 and B. When rod outer segments are flash illuminated, a proton is rapidly bound by each rhodopsin molecule (*17, 18*), and this binding probably occurs during the transition from meta I to meta II (intermediate A to intermediate B) (*18*). If the reversible binding of this proton is an oriented process, responses of similar time course and opposite polarity such as R2 and B could result.

In summary, the early RP is generated as rhodopsin proceeds through the initial stages of bleaching, the first phase of the early RP being generated less than 0.5 μsec after the stimulating flash. Three new electrical responses have now been elicited from the photoproducts formed in the eye after an initial exposure to light. These photoproducts are almost certainly the three bleaching intermediates A, B, and C of rhodopsin observed by Hagins in the intact rabbit eye. The first and second of the photoproduct responses, responses A and B, appear to arise from the photoreversal of the two reactions which generate R1 and R2, the two phases of the early RP. The close correspondence between all these electrical responses (R1, R2, A, B, and C) and the photoreactions of rhodopsin suggests that each response is generated by the net displacement of charge in the pigment molecule as it responds to the absorption of a photon.

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16. On making these identifications, it becomes possible to reinterpret the initial observations of the response discovered by Arden, Ikeda, and Siegel (*13*). These authors assumed that the fast, negative response they observed was a single response generated by both meta I and meta II. However, they observed this response in two different types of experiments. In one experiment, the eye was cooled to −1°C and then exposed to tungsten light; in the other, the eye was exposed to light before it was cooled. In both cases, on delivering the first stimulus flash, they observed a fast negative response. It now appears, from my results, that in the first case only response A should have been present, whereas, in the second case, response C should have been predominant. Since both response A and response C are fast negative responses, it would be difficult to distinguish between them with these experiments. In addition, response B, which is positive, would not be distinguished from R1.
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Rhodopsin: Responses from Transient Intermediates Formed during Its Bleaching

Abstract. *Fast electrical responses elicited from the transient intermediates in the bleaching of rhodopsin have been investigated in the retina of the albino rat. In the experiments we used two-flash stimuli triggered sequentially with a continuously variable time delay between them. At room temperature the potential is biphasic in wave-form. The dominant, corneal-positive component is preceded by a small, corneal-negative component. Cooling the retina to about 0°C suppresses the positive component and isolates the faster, negative component. Experiments with color filters show that these two components display different spectral sensitivities and, hence, suggest that each of them is produced by a different photoproduct of bleaching.*

The early receptor potential is a fast electrical response elicited from the vertebrate retina if the retina is presented with an intense flash stimulus (*1, 2*). The potential originates in the photoexcitation of visual pigment molecules and consists of two separate components of opposite polarity (*2, 3*). These two components display different temperature dependencies and the earlier, positive component can be isolated by cooling (*3*). Recently Arden and Ikeda (*4*) reported that in a rat eye which has been illuminated with tungsten light still another response is observed. The response is corneal negative and precedes the positive component of the early receptor potential. This potential is associated with photoregeneration of the visual pigment from one or more of the intermediates in the bleaching of rhodopsin. They suggested that both metarhodopsin I and II are involved in the production of this potential, although the contribution of metarhodopsin I apparently is relatively small (*5*). We now present evidence that a similar potential obtained from the rat retina by quite different techniques displays a biphasic wave form at room temperature and that each component of the potential appears to originate from a different photoproduct of bleaching.

The stimulus source consisted of two 60-joule xenon lamps (Honeywell Strobonar 65C) triggered sequentially with a continuously variable time delay between the two flashes. The two flashes were focused on the retina by simple lens arrangements, the optic axes forming an angle of about 24° with each other. This arrangement allowed investigation of the potential associated with photoregeneration at precisely timed intervals following the bleaching flash (first flash).

Throughout this study the eyes of the albino rat (Wistar strain from Purdue colony) were used. The eyes were enucleated from fully dark-adapted animals and hemisected around the equator. The vitreous half of the eye was discarded, and experiments were performed on the posterior half spread on a piece of moist filter paper.

If the hemisected eye is exposed to a series of two-flash stimuli (bleaching and test flashes), the response to the second flash (test flash) in the two-flash sequence takes on a distinct wave-form. The responses to the two-flash stimuli obtained at 23° and 0°C are displayed in Fig. 1. The response to the test flash (second flash) elicited at room temperature is biphasic (Fig. 1A). Cone (*6*) was the first to note that a biphasic potential of polarities opposite to those of the early receptor potential can be elicited from a thoroughly light-adapted eye. In contradistinction to the early receptor potential, the dominant component of this response is corneal positive with a small corneal-negative component preceding the positive component. Apparently the faster, corneal-negative component corresponds to the "photoreversal potential" reported by Arden and Ikeda (*4*). Arden and Ikeda (*4*) and Arden, Ikeda, and Siegel (*5*) have demonstrated that the negative component of this potential is associated with photoregeneration from photoproducts of bleaching. Our observations suggest that both components are associated with photoregeneration. For convenience we shall refer to the response as the "reverse potential."

The reverse potential displays a temperature dependence similar to that of the early receptor potential. With a flash separation of about 12 msec the positive component of the potential can be observed conveniently if a bleaching flash is delivered to the retina about 1 minute before the administration of the two-flash sequence (*7*). At a temperature of 23°C the potential obtained in this way is clearly biphasic (Fig. 1A). On the other hand, if a similar sequence of operations is performed at about 0°C, only the fast negative component is present (Fig. 1B). Indeed the initial bleaching flash is not needed to produce the negative component of sizable amplitude. It is readily produced when the two-flash stimuli are delivered to a completely dark-adapted eye at 0°C, providing the flash interval does not exceed about 100 msec. The responses obtained from a dark-adapted eye with a flash separation of 12 msec are shown in Fig. 1C. Thus, at about 0°C the slower positive component is abolished, and the negative component is isolated. At physiological temperature, on the other hand, Arden, Ikeda, and Siegel have shown that the presence of the reverse potential manifests itself