Rapid Dark Recovery of the Invertebrate Early Receptor Potential

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ABSTRACT The recovery in the dark of the early receptor potential, as a direct manifestation of the state of the visual pigments, has been studied by intracellular recording in the ventral photoreceptors of *Limulus* and lateral photoreceptors of *Balanus*. The recovery is exponential with $1/e$ time constants of about 80 ms at 24°C for both preparations and 1800 ms at 4°C for *Balanus*. The 24°C rate extrapolates to total recovery of the pigment within 2 s. The later part of the dark adaptation of the late receptor potential, which may take from seconds to minutes in these preparations, appears thus to be unrelated to the state of the pigment.

INTRODUCTION

In vertebrates, the dark adaptation of the visual system has a time scale of minutes. During the later stages of this adaptation, the logarithms of the sensitivities at threshold of various measures—psychophysical, rod electroretinographic, and ganglion cell activity—appear to follow the same time-course as the regeneration of the rhodopsin, determined photometrically (Dowling and Ripps, 1970, summarize the evidence on this correlation; also see Hamdorf, 1970, and Alpern et al., 1971). The recovery of the early receptor potential (ERP) (Brown and Murakami, 1964) also follows the same time-course (Berson and Goldstein, 1970; Cone and Cobbs, 1969) and this, together with the observation that the ERP is proportional to the light intensity at low intensities (Cone, 1964), suggests that the ERP is a good measure of the fraction of pigment unbleached.

A time scale for recovery of the late receptor potential (LRP) and neural response comparable with that of the vertebrate dark adaptation has been shown for *Limulus* lateral eyes (Benolken, 1962; Hartline and McDonald, 1947). Recently, dark adaptation has been studied in the *Limulus* ventral eye by Fein and DeVoe, 1973. Fast and slow components with $1/e$ decay times of 20 s and 4 min, respectively, were noted. In the *Balanus* lateral eye, our own

observations indicate adaptation times of the same order, though quantitatively different. The regeneration of invertebrate pigment has not been quantitatively examined, however. (Hubbard and St. George, 1958, report "rapid" regeneration in the squid in vivo. Tsukahara and Tasaki, 1972, show ERP recovery in an isolated octopus retina in less than 100 s; and Brown and White, 1972, saw *no* in vivo regeneration in the mosquito under their experimental conditions.) We report here a measurement of the recovery characteristics of the early receptor potential recorded intracellularly in the ventral eye of *Limulus* (ERP previously recorded by Brown, Murray, and Smith, 1967) and in the lateral ocellus of the barnacles *Balanus amphitrite* and *eburneus* (no previous ERP recording; LRP recorded by Gwilliam, 1965; Brown et al., 1968, 1969, 1970, 1971; Koike et al., 1971, and Shaw, 1972).

Recovery of a pigment is defined as a return to the initial dark-adapted condition. The *Limulus* ventral eye visual pigment appears to be a closed system with a single stable state and multiple thermally unstable states and thermal transitions (see following article). In such a system recovery will be approximately exponential with the rate constant of the slowest thermal transition, if the latter is common to all return pathways. We show in the following article that the barnacle visual pigment, however, has two thermally stable states, the "495" and "532" states (in addition to several unstable states). Activation of these stable states induces different ERP shapes, and the state populations which remain after dark adaptation depend on the wavelength of the preceding stimulation. Recovery in this case is defined as a return to the original population distribution. This requires that all stimuli be "neutral," that is of the same color as one another and as the preceding adaptation. In this system, the population of each state will recover exponentially with the rate constant of the slowest thermal transition leading to that state if the latter is common to all pathways returning to that state. We will show that the ERP shape is approximately constant during the later part of the recovery and hence that the two stable state populations recover at roughly the same rate. We will therefore quote only a single recovery constant for the barnacle as for the *Limulus* ventral eye.

We include in this report a section showing that the fast response we observe in these preparations and call the ERP has in detail the same characteristics as does the ERP in vertebrates.

METHODS

Limulus (carapace diameter 15–20 cm) was obtained from Woods Hole and the Gulf Coast of Florida, and *Balanus* (8–15 mm diameter) from Haifa and Eilat, Israel and Woods Hole, Mass. Isolated photoreceptors with several millimeters of associated nerve (attached to the optic lobe in *Limulus*) were excised in ordinary light and mounted in seawater, on a Peltier cell for controlling temperature, which was

monitored by a remote-reading thermistor. Barnacle saline, hypertonic KCl, Na-free saline (Brown et al., 1969), and various concentrations of glutaraldehyde and formaldehyde in seawater were also used as media. All recordings were intracellular, using 6–12 MΩ 2M KCl-filled micropipettes. The resting potential was between 30 and 50 mV, inside negative. Occasionally in *Limulus* and fairly often in *Balanus* this resting potential quickly disappeared spontaneously leaving less than 1 mV. This accompanied a decline in cell resistance to well below 1 MΩ, and in cell time constant to a few milliseconds, and a nearly complete disappearance of the LRP. The ERP was most easily measured in such instances, though the measurements were approximately confirmed in the presence of the LRP. Most of the *Limulus* preparations were treated with ½% Pronase (Calbiochem, Los Angeles, Calif.) (5 min), but it was possible to penetrate the *Balanus* receptors without treatment by using a corneal approach. Responses were directly photographed from an oscilloscope or averaged on a Computer of Average Transients (C.A.T.).

The light source was a quartz-iodide lamp. It was critically focused by two f-1 lenses onto a 0.5 mm diameter 90 cm light guide with output end within 0.3 mm of the photoreceptor. A loudspeaker-mounted shutter (light rise and fall times less than 1 ms) was located at the entrance to the light guide. A KG-3 heat filter, and Oriel metal-coated neutral density filters (Oriel Optics Corp., Stamford, Conn.) as needed, were inserted in the parallel beam between the two lenses. The maximum light intensity at the cell, measured by a radiometer and calibrated interference filters, was about 1×10^{16} photons per cm^2 per s per nm at 550 nm.

In order to determine the rate at which a given light runs through the pigment population, we have examined the response to a test flash presented a short time (with respect to the recovery time) after various amounts of "bleaching" light. The following article (Minke et al., 1973) shows how we can test the two states of the barnacle pigment separately by (*a*) adapting the cells to red light, and bleaching and testing them with white light ("495" state) and (*b*) adapting them to blue light and bleaching and testing them with red light ("532" state). The *Limulus* cells were adapted, bleached, and tested with white light. The test response amplitude was reduced to $1/e$ of its value in the dark by maximum white bleaching lights of duration in the range 3–7 ms in all three cases (extrapolating from red to white bleaching for the "532" barnacle state, using the absorption spectrum of this state as shown in the following article).

RESULTS

Identification of the Fast Response as an Early Receptor Potential

We show that the fast response which precedes the LRP has all of the main accepted characteristics of the visual pigment early receptor potential (Cone and Pak, 1971). In all of these characteristics (except the last) the ERP differs grossly from the LRP (Arden, 1969).

1. THE ERP HAS A VERY SMALL LATENCY Fig. 1 shows the fast response under various conditions (see legend). The latencies are always small compared with LRP latencies. The latencies of the fast response in traces E, F,

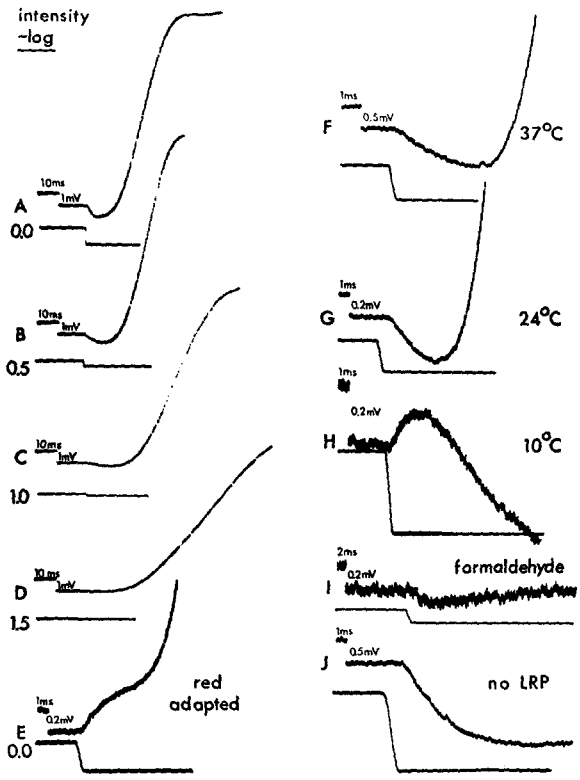

FIGURE 1. Characteristics of the ERP. This figure is designed to confirm the identification and illustrate the characteristics of the early receptor potential (ERP) in the barnacle photoreceptor. All of the responses are from *B. amphitrite*, but no qualitative differences from *Limulus* or *B. eburneus* were found, except that the phenomenon shown in trace E was not found in *Limulus*. Traces A, B, C, and D show the response at 24°C to white light of log I = 0.0, −0.5, −1.0, and −1.5, respectively, relative to the maximum intensity available. Note that the response is made up of a small fast negative (hyperpolarizing) component, the ERP, whose amplitude is strongly dependent on light intensity, and a slower positive part, much more weakly intensity dependent, the LRP. For clarity, the LRP has been reduced for this example by strong preceding light adaptation. Trace G is the response to maximum white light at 24°C but on a faster time scale (and in another cell). The fast negative response has an apparent latency of about 1 ms. Trace F shows that this drops to 0.3 ms at 37°C. Note that the sweep speed in F is twice that in G and H. Trace H shows that at 10°C a positive component emerges with a latency less than 0.3 ms while the time to negative peak (as in Fig. 3) and the latency of the LRP have become longer than the sweep. Trace E at 24°C shows that previous adaptation of a *Balanus* cell to red light isolates a positive response (see following article) whose latency is also small, less than 0.4 msc. Trace I is the result of fixation of the cell at 24°C in 0.6% formaldehyde. By comparison with trace G, one notes the disappearance of the slow response and the survival and speeding up of the fast response. Trace J shows a cell also at 24°C in which the slow response had disappeared spontaneously (compare trace G). The output of a photocell placed near the photoreceptor is displayed (with various gains) under each response. The height and duration of the initial calibration step are shown for each response. From these observations it is clear that the fast response is an ERP (see text).

and H, if any, are all less than about 0.4 ms. Traces G, I, and J do show finite latencies of around 1 ms; however, Pak and Cone (1964) and others have interpreted the disappearance of the fast positive phase with increasing temperature (see section 2, below) as arising from the speeding up of a thermal process responsible for the negative phase. It seems likely, therefore, that even this small latency actually arises from the cancellation of the fast positive phase with the early part of the larger, but initially more slowly rising, negative phase.

2. THE ERP IS PURELY NEGATIVE AT HIGH TEMPERATURES (TRACES F, 37°C AND G, 24°C) AND DEVELOPS AN INCREASING INITIAL POSITIVE PHASE WITH DECREASING TEMPERATURE (TRACE H, 10°C) The exceptional case of the purely positive response after red adaptation in *Balanus* (trace E) but not in *Limulus* will be discussed in the following article.

3. THE ERP SURVIVES FIXATION The ERP survives fixation (trace I, formaldehyde; similar response with 0.6% glutaraldehyde solution) which destroys the slow response and speeds up the fast response apparently because of a decreased membrane resistance. At high glutaraldehyde concentrations (5%) the response develops, even at room temperature, a fast positive phase, similar to trace H, though of decreased amplitude.

4. THE AMPLITUDE OF THE ERP IS PROPORTIONAL TO LIGHT INTENSITY UP TO SATURATION Saturation occurs at intensities sufficient so that most of the pigment molecules have absorbed at least one photon. Traces A, B, C, and D show the response at 24°C to white light of intensity 0, 0.5, 1.0, and 1.5 log units, respectively, below maximum available intensity. The large slow positive (depolarizing) response is the LRP. The LRP amplitude is near saturation in the range illustrated, but was found to depend roughly logarithmically on light intensities below this range (see Brown et al., 1971). The fast response, on the other hand, is closely proportional to light intensity in the range illustrated.

Fig. 2 shows the dependence on light intensity of the amplitudes of the positive and negative peaks of the biphasic fast response of a white-adapted cell at 8°C (similar to trace H) and of the positive peak of the response of the same cell when red-adapted (trace E). For these measurements a cell was used where the LRP had disappeared spontaneously (as in trace J). A log-log plot allows an extended range of intensities to be displayed. On such a plot, a straight line with slope 1 corresponds to a linear dependence. For all three response phases the points appear to fit or approach a straight line with slope 1 up to intensities where the light begins to run through an appreciable fraction of the pigment population (see Methods) by the time the response-phase peak is reached. The negative-phase peak occurs, at this low temperature, a longer time after the onset of the stimulus (which remains on) than does the

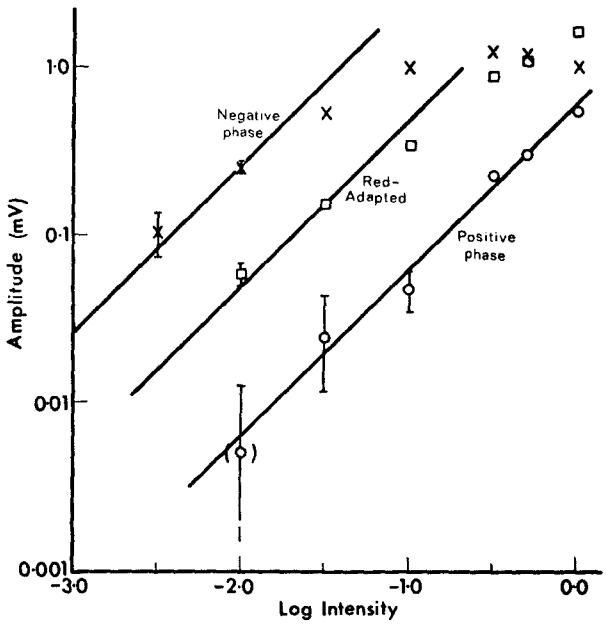

FIGURE 2 The linearity of the fast response. *Balanus amphitrite*, 8°C. Abscissa: light intensity relative to maximum (see Methods), logarithmic scale. Ordinate: the amplitudes (in millivolts, logarithmic scale) of the positive and negative phases of the biphasic fast response (trace H of Fig. 1) and of the positive fast response of the same cell after adaptation to red light (trace E of Fig. 1). Estimated experimental error limits are shown where they exceed the size of the symbols. Straight lines with slope 1 are drawn to fit all points of the positive-phase data and the low-intensity points of the negative-phase and red-adaptation data. See text.

red-adapted peak, so saturation occurs in the former at lower intensity but after equal amount (intensity times time-to-peak) of light. The positive-phase peak is so fast that no saturation is expected, or seen, in the accessible intensity range.

5. THE ERP IS INSENSITIVE TO IONIC MEDIUM In hypertonic KCl and in Na-free saline, the LRP was much reduced while the fast response remained relatively unchanged.

6. THE ERP HAS THE SAME ACTION SPECTRUM AS THE PHYSIOLOGICAL RESPONSE We show in the following articles that the positive and negative phases of the fast response have action spectra (measured in red-adapted and white- or blue-adapted cells, respectively) which match those of different aspects of the LRP response phenomena.

By these various criteria, we identify the fast response in all of these observations as an early receptor potential, that is, a direct electrical manifestation of the successive changes undergone by a visual pigment after photon absorption.

Recovery Measurements

The measurements within any set were made by repeatedly presenting to the cells pairs of light pulses of fixed length and intensity and variable separation, at repetition intervals ranging from 1.25 s to minutes. The resulting recovery time constants showed no dependence on this interval, nor on the choice of length, intensity, or wavelength of the pulses. In every case the cell was previously adapted to the same color as the test pair to ensure that no net change of pigment populations occurred due to the pulse pair. Results were obtained both from averaged records and from single traces.

Sample recordings are shown in Fig. 3 for *Limulus* at 7° and 19°C, and for *Balanus* at 4° and 24°C. Since the shape of the response to the second pulse is very similar to that of the response to the first, except for very short dark intervals, the amplitude of the negative excursion, with respect to the base line immediately preceding each response, was a convenient recovery criterion. The uncertainty introduced by the response distortion at short dark intervals is small. Each point on the recovery curves (Fig. 4) is taken from a single trace like those of Fig. 3. The negative amplitude of the second response (A_t) is subtracted from that of the first response (A_{max}) and the difference divided by the amplitude of the first response. The result, $(A_{max} - A_t)/A_{max}$, is plotted

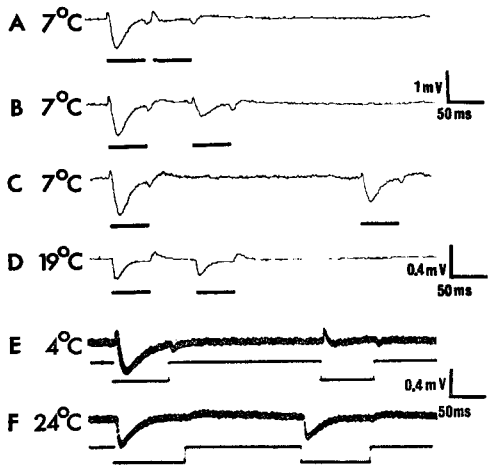

FIGURE 3. ERP responses to pairs of stimuli. The top three traces show early receptor potential responses to successive 50-ms light pulses of equal strength and duration (bars) recorded in the ventral eye of *Limulus* at 7°C. The first two curves are averages of 64 runs with a repetition sycle of 1.25 s and the third is an average of 16 runs with a repetition cycle of 5 s. Note the growth of the second response, relative to the first, with dark interval. The fourth trace (64 runs, repetition cycle 1.25 s) is in the same cell at 19°C. The response is much smaller than at 7°C due to a decline in membrane resistance. The last two traces are single-sweep recordings in a *Balanus amphitrite* cell at 4° and 24°C, respectively. The three sets of calibration axes apply to traces A, B, and C; trace D; and traces E and F, respectively.

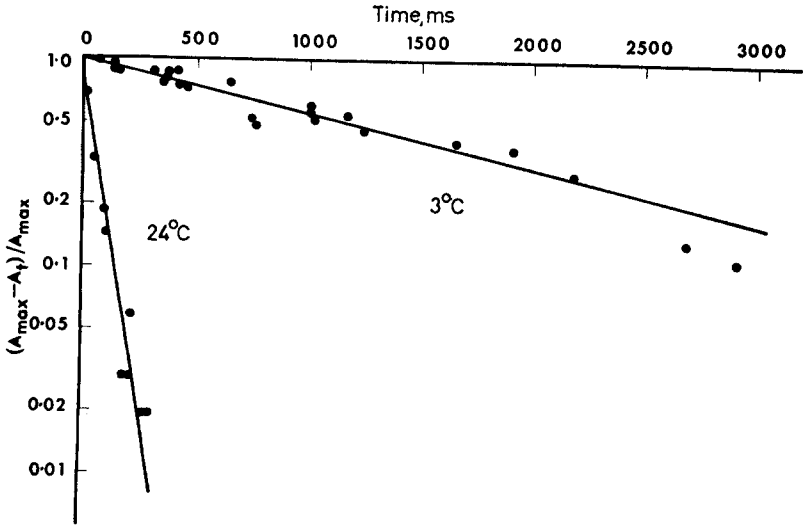

FIGURE 4. The ERP dark recovery. The logarithm of the difference between the sizes of the negative peaks of the early receptor potential responses to a pair of light pulses, relative to the size of the response to the first pulse, as a function of time in the dark between the two pulses of the pair. *Balanus amphitrite*, 3° and 24°C. The straight lines represent exponential recoveries with 1700 and 75 ms time constants, respectively.

in Fig. 4 on a logarithmic scale against the dark interval between pulses. For an exponential recovery of time constant τ,

$$A_t = A_{max}[1 - \exp(-t/\tau)], \quad \text{or} \quad \log \frac{A_{max} - A_t}{A_{max}} = -t/\tau.$$

Fig. 4 shows the recovery results obtained in two *Balanus* experiments. To a good approximation, the points fall on straight lines, indicating an exponential recovery. Dark times of up to 30 min were used to search for slower components of the recovery, but none were found.

For light stimuli too short or dim for the response to reach a steady state (too short or dim to saturate the pigment), the line is expected, and experimentally is found, to extrapolate to a number less than 1, since part of the pigment remains unaffected by the first pulse. The recovery slopes were found to be the same in these cases.

The $1/e$ time constants for exponential recovery were obtained by averaging the results of several experiments over small temperature ranges around 24° and 4°C, all extrapolated to these temperatures. The results are: 80 ms for both *Limulus* and *Balanus* at 24°C, 1800 ms for *Balanus* at 4°C (no good low temperature measurements were made in *Limulus*). All measurements fell within 30% of the means, and in a given preparation within 10% except in one experiment where a drift of 20% was apparently observed in the time constant during the experiment. No appreciable differences between the two closely related *Balanus* species were found, so results of the two were pooled.

DISCUSSION

If the $1/e$ recovery time constant continues to decline with increasing temperature at the same rate between 24° and 37°C as between 4° and 24°C, it will reach 11 ms at 37°C. This contrasts with human and monkey cone and rod pigment regeneration time constants of roughly 2 and 5 min, respectively (see summary in Berson and Goldstein, 1970, also Goldstein, 1969; Goldstein and Berson, 1969; and Alpern et al., 1971), and a frog cone pigment regeneration time constant of 36 s (Taylor, 1969). These values are about four orders of magnitude more than those found in the present preparations. This may be related to the tendency of invertebrate visual pigments not to proceed to dissociation (Brown and Brown, 1958; according to Hubbard and Wald, 1960, however, the pigment *does* dissociate in *Limulus*). One wonders what evolutionary advantage there is, if any, in the much slower pigment recovery in vertebrates. This difference is *not*, however, substantially reflected in the LRP recovery speeds (see Introduction). In fact, if the invertebrate pigment recovery continues exponentially, the pigment will be completely regenerated (that is, it will be down to its last molecule) in about 2 s at room temperature. The LRP recovery in both preparations is slower than this and is also different in the two preparations (see Introduction) despite the equality of the ERP recovery speeds. An improbable interpretation would be that there exists in both preparations a pigment state to which the pigment totally converts in 2 s; which decays slowly to the normal state; and activation of which gives the same ERP with the same action spectrum as the normal state (since we found no wavelength dependence of the recovery time) but no (or suppressed) LRP. A more likely interpretation is that at least the later part of the dark adaptation of the receptor potential in these invertebrates must be unrelated to the state of the pigment.

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